THE PRODUCTION OF PODOPHYLLOTOXIN AND RELATED CYTOTOXIC LIGNANS BY PLANT CELL CULTURES

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te Tiel

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CHAPTER 1

GENERAL INTRODUCTION

PODOPHYLLOTOXIN AND RELATED LIGNANS

HISTORY

Since ancient times, several lignan-producing plants containing podophyllotoxin and related compounds have been used for the treatment of certain malignancies. *Juniperus sabina* has the most ancient history with respect to successful cancer treatment. As early as in the first century A.D., the needles of *J. sabina* were said to remove unhealthy granulations. Generally, dried needles, called savin, or the derived oil was used. In 47 A.D. Scribonius Largus wrote that savin oil was used to soften "hard female genital parts". Later references indicated the use of savin to treat uterine carcinoma, venereal warts and polyps. At present, we know that the pharmacological activity of *J. sabina* needles is to be ascribed to the lignans desoxypodophyllotoxin and podophyllotoxin (Hartwell et al. 1953, 1958). The Leech book of Bald, 900-950 A.D., an early English medicinal book, has reported on the use of root extracts of *Anthriscus sylvestris*, which contain several podophyllotoxin derivatives, in ointments to cure cancer (Cockayne 1961).

The plant species that are currently used for the extraction of podophyllotoxin, are *Podophyllum peltatum* and *P. hexandrum*. *P. peltatum* is indigenous to the Eastern part of the United States of America and Canada. *P. hexandrum* is found in the higher parts of the Himalaya mountains and also referred to as the Indian *Podophyllum* (Chatterjee 1952; Hartwell et al. 1953, 1958; Meijer 1974; Horwitz and Loike 1977; Gupta and Sethi 1983; Ayres and Loike 1990). The natives of the Himalayas as well as the American Indians, independently discovered that extracts of rhizomes possessed a cathartic action. The Indians introduced podophyllin, a resin obtained by ethanolic extraction of the *Podophyllum* roots and rhizomes, to colonists for use as a cathartic, an anthelmentic and misuse as a mortal poison. The colonists in turn used this resin as an emetic and a cholagogue also. Podophyllin was included in the first U.S. Pharmacopoeia, dating from 1820, as a catharctic and cholagogue. Because of its severe toxicity the drug was removed from the 12th edition of this Pharmacopoeia, that appeared in 1942 (Horwitz and Loike 1977). In the same year however, it was reported that venereal warts (Condyloma acuminatum) could be selectively destroyed by the topical application of podophyllin

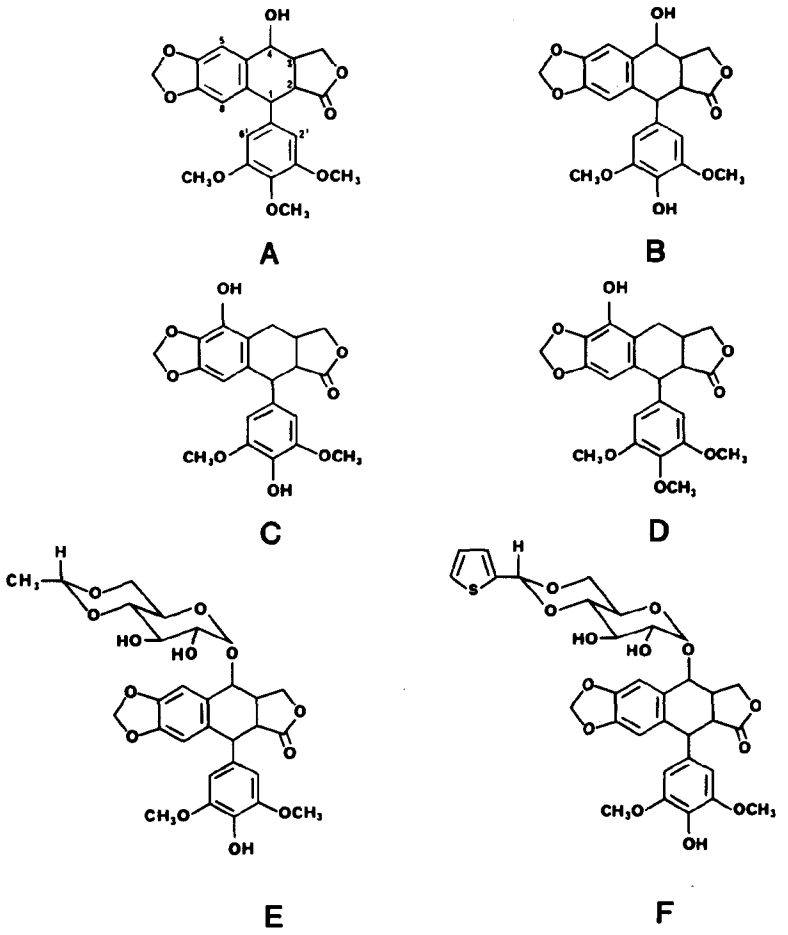

Fig. 1. Chemical structures of podophyllotoxin (**A**), 4'-demethylpodophyllotoxin (**B**), α-peltatin (**C**), β-peltatin (**D**), etoposide (**E**) and teniposide (**F**).

resin. Nowadays, this resin is still the drug of choice in the treatment of this disease (Ayres and Loike 1990).

It was not until 1947 that the first reports appeared dealing with the inhibition of growth of cancer in animals by podophyllin (Horwitz and Loike 1977). The major constituents in podophyllin, the lignans podophyllotoxin, 4'-demethylpodophyllotoxin, α- and β-peltatin (Fig. 1), induce severe gastrointestinal side effects, as is also the case with their glucosides, thus limiting their clinical use (Stähelin and Von Wartburg 1989, 1991). With the aim to find clinically applicable drugs, related to these naturally occurring lignans, series of glycoside and aglycone derivatives have been prepared by chemical synthesis by Sandoz Pharma. Especially, condensation products of *Podophyllum* glucosides with benzaldehyde were of particular interest. Based on this finding two new lignans were

developed in 1965-1966 by performing aldehyde condensations with 4'-demethylepipodophyllotoxin-4-O-β-D-glucoside, resulting in etoposide (VP-16-213, Vepesid®) and teniposide (VM-26, Vumon®), Fig. 1, (Stähelin 1979, 1973). At present, etoposide and teniposide are clinically applied as chemotherapeutics that are particularly effective against testicular and small cell lung cancer (Van Maanen 1988). The natural source for the semi-synthesis of these cytostatics is podophyllotoxin extracted from the rhizomes of *Podophyllum* species. After Sandoz Pharma had commercialized teniposide in several countries in 1976, etoposide and teniposide were licensed out to Bristol-Myers in 1978. The latter firm introduced etoposide on the drug market in the United States of America in 1983 (Stähelin and Von Wartburg 1991).

OCCURRENCE OF PODOPHYLLOTOXIN AND SOME RELATED COMPOUNDS IN PLANTS

Lignans occur widespread throughout the plant kingdom (Cole and Wiedhopf 1978). The biological functions of plant lignans are not clear, although the high toxicity of most lignans for living organisms suggest a function with relation to the plant host-defence system (Ayres and Loike 1990). Podophyllotoxin, however, is restricted to only a few plant species, listed in Table 1. From this table it can be seen that podophyllotoxin occurs mainly in the families Berberidaceae and Cupressaceae. The highest podophyllotoxin contents are found in the rhizomes of *Podophyllum hexandrum* and in the needles of *Callitris drummondii*. However, it has to be considered that the podophyllotoxin yields of *P. hexandrum* vary strongly with the location of cultivation and the season of collection. It is maximal when the plant has reached the flowering stage (Chatterjee 1952). Next to podophyllotoxin several other lignans have been detected in the listed plant species.

Recently, trace amounts of podophyllotoxin-β-D-glucoside have been found in the roots of *L. flavum* cv. 'Compactum' (Broomhead and Dewick 1990b). The lignan content of *L. flavum* roots is interesting because high quantities (3.5-4.1% on a dry weight basis) of 5-methoxypodophyllotoxin, present as aglucone or glucoside, are comparable with the amounts of podophyllotoxin in *P. hexandrum* rhizomes. The aerial parts of *L. flavum* also contain 5-methoxypodophyllotoxin, 10-50% of the content in the roots have been reported (Broomhead and Dewick 1990b; Wichers et al. 1991).

5-Methoxypodophyllotoxin and its glucoside have also been detected in roots of *Linum capitatum* and in the needles of *Juniperus sabina*, 0.25% and 0.001% on a dry weight basis respectively (Broomhead and Dewick 1990; San Feliciano et al. 1990).

Table 1. Distribution of podophyllotoxin in the plant kingdom.

Species	Family	% Podophyllotoxin (dry weight)	Source	Reference
Diphylleia cymosa	Berberidaceae	0.12-0.54	leaves	Broomhead and Jackson 1990a
Diphylleia grayi	Berberidaceae	1.27	roots	Broomhead and Jackson 1990a
Podophyllum hexandrum	Berberidaceae	1-4.9	rhizome	Broomhead and Jackson 1990a; Fay and Ziegler 1985; Chatterjee 1952
Podophyllum peltatum	Berberidaceae	0.25-1.0	rhizome	Broomhead and Jackson 1990a; Fay and Ziegler 1985
Podophyllum pleianthum	Berberidaceae	0.14	rhizome	Broomhead and Jackson 1990a
Podophyllum versipelle	Berberidaceae	0.32	rhizome	Broomhead and Jackson 1990a
Callitris drummondii	Cupressaceae	1.4 [a]	needles	Kier et al. 1963
Juniperus lucayana	Cupressaceae	0.10	needles	Hartwell et al. 1953; Hegnauer 1962
Juniperus sabina	Cupressaceae	0.20	needles	Hartwell et al 1953; Hegnauer 1962; San Feliciano et al. 1989a, 1990
Juniperus scopulorum	Cupressaceae	0.17	needles	Hartwell et al. 1953; Hegnauer 1962
Juniperus silicicola	Cupressaceae	0.04	needles	Hartwell et al. 1953; Hegnauer 1962
Juniperus thurifera	Cupressaceae	1.3×10^{-4} [b]	needles	Hegnauer 1962; San Feliciano et al. 1989b
Juniperus virginiana	Cupressaceae	0.10	needles	Hartwell et al. 1953; Hegnauer 1962
Linum album	Linaceae	4.5×10^{-4} [b]	whole plant	Weiss et al. 1975
Linum flavum 'Compactum'	Linaceae	traces [a]	roots	Broomhead and Dewick 1990b
Polygala polygama	Polygalaceae	0.08	whole plant	Hokanson 1978

a Present as β-D-glucoside.
b On a fresh weight basis.

BIOSYNTHESIS

From a chemical point of view, podophyllotoxin and related compounds belong to the lignans. Lignans are defined as dimerization products of two phenylpropane units, which are linked by the β-carbon atoms of their side-chains (Weinges et al. 1978; MacRae and Towers 1984; Pelter 1986; Dewick 1989). They originate from the phenylpropanoid biosynthesis route, in which also lignin is formed. Lignans are produced stereospecifically under enzymatic control (Pelter 1986; Frias et al. 1991), whereas lignin is an at random produced compound consisting of hundreds of phenylpropane units (Janshekar and Fiechter 1983). At present, there is no evidence whether lignans represent intermediates in the lignin biosynthesis, or whether their formation represents a competitive pathway in the utilization of lignin precursors (Dewick 1989).

The photosynthetic assimilation of CO_2 by plants results into the production of carbohydrates, that can be metabolized via the shikimic acid pathway finally leading to the various phenylpropanoids (Janshekar and Fiechter 1983; Dewick 1989). This first part of the lignan biosynthesis is depicted in Fig. 2.

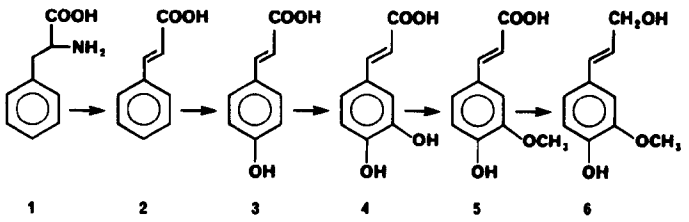

Fig. 2. Simplified scheme illustrating the biosynthesis of coniferyl alcohol (**6**) from phenylalanine (**1**), via cinnamic acid (**2**), coumaric acid (**3**), caffeic acid (**4**) and ferulic acid (**5**).

The cinnamic acid derivatives may be stored as their glucosides or be converted into their respective alcohols by the action of dehydrogenases. After the removal of the glucosyl moiety by the action of a glucosidase, the dimerization is induced by the action of peroxidases (Ayres and Loike 1990). Recently, a rather specific peroxidase occurring in the leaves of *Bupleurum salicifolium* has been isolated and purified, it catalyses the dimerization of ferulic acid, caffeic acid or coniferyl alcohol into lignans by oxidative coupling (Frias et al. 1991). This enzyme did not exhibit activity on other phenylpropanoids, such as cinnamic acid, coumaric acid and 3,4-dimethoxycinnamic acid, that were tested as substrates in that study. The finding of an enzyme, catalysing the dimerization of phenylpropanoids through a ββ'-linkage, supports the hypothesis that

lignans are formed under enzymatic control (Frias et al. 1991). In addition, Umezawa et al. (1991) found that two coniferyl alcohol moieties were directly coupled, affording only (-)-secoisolariciresinol which is subsequently stereoselectively converted into (-)-matairesinol, using cell-free preparations from *Forsythia intermedia*. No evidence for the formation of the corresponding (+)-enantiomers was found.

Ayres et al. (1969) showed that labelled phenylalanine was taken up by *Podophyllum hexandrum* plants and incorporated into podophyllotoxin to the extent of 1.4%. In a following study (Ayres 1981), it was demonstrated that *para*-coumaric acid and phenylalanine were incorporated, 0.57% and 1.17% respectively, while tyrosine and acetate were not. In addition, Jackson and Dewick (1984a) incubated *P. hexandrum* plants with a series of phenylpropanoids. Cinnamic, ferulic and 3,4-methylenedioxycinnamic acid were incorporated at levels of 0.17, 0.05 and 1.34% respectively. In contrast, the highly oxygenated sinapic and 3,4,5-trimethoxycinnamic acid were not incorporated at all. Stöckigt and Klishies (1977) supported the idea of a late-stage oxygenation and the effective blocking of the *para*-phenolic position by methyl but not by the methylenedioxy group in the formation of lignans. Moreover, the fact that the *Bupleurum* peroxidase, isolated by Frias et al. (1991), only dimerized cinnamic acid derivatives carrying a hydroxy group at the *para*-position and a hydroxy or methoxy group at the *meta*-position, fits in this idea. Feeding of [O-$^{14}CH_3$] ferulic acid yielded podophyllotoxin with a level of labelling in the methylenedioxy moiety comparable with that in the methoxyl groups (Jackson and Dewick 1984a). This finding suggests a biosynthetic sequence involving an oxidative coupling of two similar phenylpropane precursors possessing the substitution pattern of ferulic acid. This oxidative dimerization takes place at the alcohol level. Therefore it is very likely that coniferyl alcohol is the intermediate involved (Stöckigt and Klishies 1977; Dewick 1989; Rahman et al. 1990; Broomhead et al. 1991).

As a subsequent step, coniferyl alcohol is dehydrogenated to mesomeric phenoxy radicals (Fig. 3).

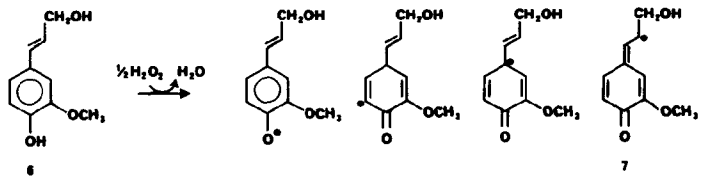

Fig. 3. Formation of radicals by the oxidation of coniferyl alcohol (**6**).

The coupling product of 2 'β-radicals' is a lignan with a diquinonemethide structure that, in turn, is metabolized by reduction, the formation of a lactone ring and the modification of the aromatic substitution pattern, to yield matairesinol (Fig. 4) (Broomhead et al. 1991). Jackson and Dewick (1984b) showed that, after the coupling of the 2 phenylpropane units, the paths to podophyllotoxin and 4'-demethylpodophyllotoxin diverge before the cyclization step occurs. Thus, 4'-demethyl lignans could not be converted into 4'-methoxy lignans.

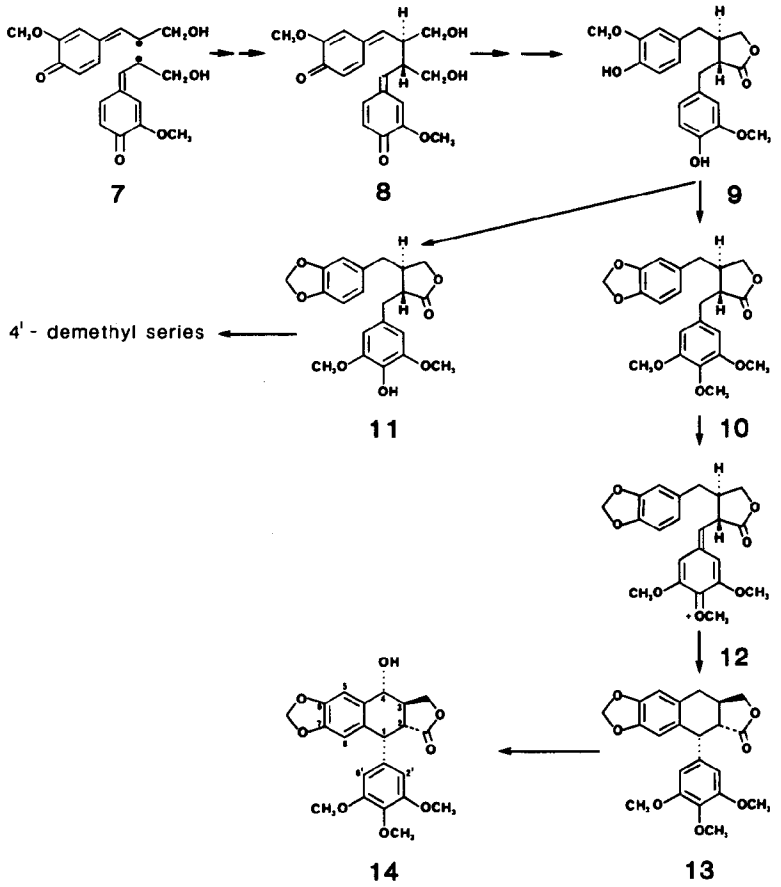

Fig. 4. Proposed biosynthetic pathway from coniferyl alcohol radicals (**7**) to podophyllotoxin (**14**) via a diquinonemethide (**8**), matairesinol (**9**), yatein (**10**), (4'-demethylyatein (**11**)), a quinonemethide (**12**) and desoxypodophyllotoxin (**13**), after Broomhead et al. (1991).

Matairesinol has been proposed to be the branchpoint compound to the trimethoxy and hydroxydimethoxy series of aryltetralin lignans in *Podophyllum* species (Kamil and Dewick 1986; Dewick 1989). Recently, feeding experiments with matairesinol in three different plant systems, *P. hexandrum*, *P. peltatum* and *Diphylleia cymosa*, have been performed (Broomhead et al. 1991). It was demonstrated in this study that matairesinol is a common precursor of the 3',4',5'-trimethoxy as well as of the 4'-hydroxy-3',5'-dimethoxy groups of *Podophyllum* lignans and it may indeed be considered as the branchpoint compound. Matairesinol is further substituted to the dibenzylbutyrolactones yatein and 4'-demethylyatein, followed by cyclization and specific modification processing of a quinonemethide resulting in the various aryltetralin lignans (Fig. 4).

Desoxypodophyllotoxin is a precursor of podophyllotoxin, this means that the 4-hydroxy group has to be introduced after dimerization and cyclization. The same has been found to be true in the 4'-demethyl series (Dewick and Jackson 1984b). Analogously, feeding experiments with labelled 4'-demethyldesoxypodophyllotoxin and desoxypodophyllotoxin using *P. peltatum* plants indicated that these compounds are most probably the precursors of α- and β-peltatins respectively, via hydroxylation at C-5 (Kamil and Dewick 1986).

The occurrence of 5-methoxypodophyllotoxin in *Linum* species indicates a difference in biosynthesis capability between the genera *Linum* and *Podophyllum*. Podophyllotoxin and β-peltatin are hydroxylation products of desoxypodophyllotoxin on the C-4 and C-5 position, respectively. The question whether 5-methoxypodophyllotoxin, the major compound in *Linum flavum*, originates from further substitution of podophyllotoxin or from β-peltatin has not yet been answered. Traces of podophyllotoxin have been found in *L. flavum* suspension cultures (Wichers et al. 1991), while traces of β-peltatin have been found in the cultivar *L. flavum* 'Compactum' (Broomhead and Dewick 1990b).

CHEMICAL SYNTHESIS

Many attempts have already been made to prepare the commercially attractive podophyllotoxin by organic synthesis (Ayres 1978; Doyle 1984; Forsey et al. 1989; Ayres and Loike 1990; Ward 1990). Although several elegant syntheses of this lignan have been described, the currently available methods for the synthesis of podophyllotoxin are very lenghty and complex. Overall yields lie in the range of 10-15%, being too low to have economical value (Doyle 1984; Ayres and Loike 1990; Ward 1990). The main problem for organic chemists is the presence of 4 chiral centers, a rigid *trans*- lactone and an axially locked aryl substituent in the podophyllotoxin molecule. In addition, even the slightest trace of base rapidly converts the *trans*-lactone into picropodophyllin, its more stable *cis*-form (Forsey et al. 1989).

Podophyllotoxin and many related lignans possess antimitotic activity. It has been found that podophyllotoxin binds to purified tubulin preparations. After the addition of podophyllotoxin, cultured cells are arrested at the metaphase because it prevents the microtubule assembly. Cells can still enter mitosis and perform a normal prophase, but the separation of the duplicated chromosomes is impaired, the cells accumulate in the metaphase with clumped chromosomes, and cell proliferation is inhibited (Horwitz and Loike 1977; MacRae and Towers 1984; Stähelin and Von Wartburg 1991). Podophyllotoxin is not the only compound that disrupts the assembly and function of microtubules. Other microtubule inhibitors, regarded as spindle poisons, include the plant constituents colchicine, taxol and vincristine-like alkaloids (Ayres and Loike 1990; Stähelin and Von Wartburg 1991).

Structure-activity relationship studies have been carried out to evaluate the spindle-poison activity of podophyllotoxin and resulted in a number of important conclusions: [1]. The C-4 hydroxy moiety is of little importance [2]. Epipodophyllotoxin, the C-4 stereoisomer of podophyllotoxin, is less effective than podophyllotoxin. [3]. A glucoside moiety at the C-4 position reduces the tubulin binding activity. [4]. Stereochemical C-2 and C-3 isomers exhibit a reduced ability to bind tubulin. [5]. The presence of the lactone ring has been shown to be very important. [6]. 4'-Demethylpodophyllotoxin has about the same effect on tubulin aggregation as podophyllotoxin (MacRae and Towers 1984).

Except for the affection of microtubule assembly, podophyllotoxin has been shown to inhibit the synthesis of DNA, RNA and proteins in cultured HeLa tumour cells. This is probably caused by the inhibition of the uptake of the nucleosides thymidine, uridine, adenosine and guanine in the cell by podophyllotoxin and many of its derivatives (Loike and Horwitz 1976a, 1976b; Gensler et al. 1977).

Chromosomal damage, caused by a series of lignans has been studied by Loike and Horwitz (1976a). Podophyllotoxin did not give DNA fragmentation, whereas 4'-demethylpodophyllotoxin did. In addition, epipodophyllotoxin and 4'-demethylepipodophyllotoxin exhibited a stronger activity. Remarkably, DNA damage was not observed when the experiments were performed with purified DNA and therefore it was concluded that cellular processes are involved.

Finally, podophyllotoxin and related lignans contain two aromatic rings linked by four carbon atoms, and these rings always contain at least two adjacent oxygen functions. Such compounds can release *ortho*-quinones, which may interfere with natural redox systems, leading to the induction of free radicals and reactive oxygen intermediates (Pelter 1986).

Other biological effects have been reported and include: antiviral activity, cytotoxicity, hepatotoxic protection, activity on the central nervous system, cardiovascular activity,

stress protection, cathartic effects and allergenicity, hypotensive effects, influence on nucleic acids and enzymes, inhibition of respiratory enzymes, insecticidal and piscicidal activity, germination inhibition, antimicrobial and fungistatic activity (MacRae and Towers 1984; Pelter 1986).

The important antiviral activity of lignans is probably a result of the inhibition of microtubule assembly, although the interference with nucleic acid metabolism may play a role in blocking the viral replication as well (MacRae and Towers 1984). A variety of lignans has been tested for antiviral activity by Markkanen et al. (1981). It was found that podophyllotoxin, desoxypodophyllotoxin and β-peltatin are most potent.

In addition, podophyllotoxin was found to be active against measles and herpes simplex type I (Bedows and Hatfield 1982). MacRae et al. (1989) found that podophyllotoxin and α-peltatin were potent inhibitors of the murine cytomegalus virus replication cyclus *in vitro*. Crude extracts of *P. peltatum* were able to reduce the effects of herpes simplex type II, influenza A and vaccinia viruses (MacRae and Towers 1984).

ETOPOSIDE AND TENIPOSIDE

CHEMICAL SYNTHESIS

Etoposide and teniposide are prepared by chemical synthesis using podophyllotoxin as the starting compound, which is extracted from the rhizomes of *Podophyllum* species. In a first step, podophyllotoxin is converted to 4'-demethylepipodophyllotoxin by the selective cleavage of the 4'-methoxy group with HBr, followed by hydrolysis and epimerization of the bromo intermediate. 4'-Demethylepipodophyllotoxin, protected as a benzyloxycarbonyl derivative, is treated with tetraacetyl-β-D-glucose in the presence of BF_3-etherate to yield the tetraacetate of 4'-benzyloxycarbonyl-4'-demethylepipodophylloxin-β-D-glucoside. The protecting groups are removed by submitting the tetraacetylglucoside to zinc acetate catalysed methanolysis. The intermediate is hydrogenolyzed to yield 4'-demethylepipodophyllotoxin-β-D-glucoside. This glucoside is condensed with acetaldehyde or thiophene aldehyde to yield etoposide and teniposide respectively (Stähelin and Von Wartburg 1989, 1991; Ayres and Loike 1990).

MECHANISM OF ACTION

Etoposide and teniposide exhibit a mechanism of action, which is different from podophyllotoxin. By the action of these semi-synthetics, cells are arrested in the late S or G_2 phase of the growth cycle, resulting in a premitotic block (Stähelin and Von Wartburg 1991).

Loike and Horwitz (1976a, 1976b) reported about the fragmentation of DNA in cultured HeLa cells after incubation with etoposide and teniposide, while the treatment of purified DNA with these compounds had no effect. Etoposide and teniposide cause dose-dependent single- and double-strand breaks in DNA. In addition, inhibition of DNA topoisomerase II has been reported. This enzyme creates and reseals double-strand DNA breaks. Etoposide and teniposide probably impair the strand-rejoining activity of the topoisomerase II, although it is not clear yet how the interaction between the drug and the enzyme actually results in cell death. Other effects induced by the two cytostatics include free radical formation and inhibition of the cell respiratory function. Furthermore, several metabolites of both drugs have been found to be cytotoxic (Clark and Slevin 1987; Holthuis 1988; Van Maanen et al. 1988). Still the mechanism by which etoposide and teniposide produce cytotoxicity is not exactly known. It may be the result of a combination of the mentioned effects (Bernasconi 1981; D'Incalci 1985; Van Maanen et al. 1988). Essential for the change from a spindle poison (podophyllotoxin) to a G_2 poison (etoposide and teniposide) are: the presence of a hydroxyl group at the C-4' atom, an epiconfiguration at the C-4 position and the presence of a glucoside moiety at position C-4, with an aldehyde condensed to the glucoside (Fig. 1; Van Maanen et al. 1988; Stähelin and Von Wartburg 1991).

CLINICAL APPLICATION AND PROSPECTS

Tumours being particularly sensitive to etoposide are: small cell lung and testicular cancer, Kaposi's sarcoma, lymphoma and acute lymphocytic leukemia, Ewing's tumour, gestational germ tumours, Hodgkin's disease and neuroblastoma (Issell et al. 1984). Etoposide has proven to be a very effective cytostatic agent, especially in combination with cisplatin. A complete remission of approx. 75% of the treated patients who suffered from small cell lung cancer or testicular cancer, and overall responses of 90% have been reported (Issell et al. 1984; Henwood and Brogden 1990; Aisner and Lee 1991). It has to be considered however, that a key problem in the treatment of patients that suffer from malignant tumours exists, namely the development of resistance to these therapeutics (D'Incalci 1985; Clark and Slevin 1987; Stähelin and Von Wartburg 1989; Henwood and Brogden 1990). After years of preclinical and clinical testing it appeared that no significant therapeutic difference exists between etoposide and teniposide (Issell et al. 1984; Ayres and Loike 1990).

Currently, research is focussed on the development of new epipodophyllotoxin derivatives with an improved anti-tumour activity and selectivity in combination with a decreased toxicity (Stringfellow and Schurig 1987; Holthuis 1988; Ayres and Loike 1990). Etoposide and teniposide are recognized anti-tumour agents, which full potential has not

been completely explored yet. Their synthesis from podophyllotoxin represents a model in the development of new drugs from natural products. Stähelin and Von Wartburg (1989) stated that the clinical evaluation and application of these two drugs will make progress and that the search for *Podophyllum* compounds that possess a higher clinical utility continues.

SCOPE OF THE THESIS

It is to be expected that the chemical synthesis of podophyllotoxin and related compounds will not be able to fulfill the commercial needs in the foreseeable future and therefore, the production of these compounds will ultimately depend on the availability of the raw plant materials. The supply of *Podophyllum* plants has its limitations, since the occurrence of the plants is scarce and they have a long, 5-7 years, juvenile phase as well as poor reproduction capacities (Rust and Roth 1981; Chuang and Chang 1987). *P. hexandrum* has even become a threatened species due to the intense collection of this plant from the Himalayan forests and the lack of organized cultivation (Gupta and Sethi 1983). Consequently, the first attempts have already been made to conserve this medicinally important plant using *in vitro* multiplication methods (Arumugam and Bhojwani 1990; Fujii 1991).

Due to the time-consuming collection of *Podophyllum* plants and the rather difficult isolation from the rhizomes, podophyllotoxin is an expensive starting compound for the chemical synthesis into etoposide and teniposide. The bad perspectives for the availability of podophyllotoxin may result in unsolvable problems in the production of anti-tumour agents.

The biotechnological production of podophyllotoxin and related compounds using plant cell cultures can be considered as an alternative to overcome the difficulties in the supply of these compounds.

At the start of this study in 1988, only two studies had been performed with tissue cultures derived from plant species as mentioned in Table 1. Kadkade (1981,1982) initiated callus cultures from the rhizome, leaf and root segments of field-grown plants of *Podophyllum peltatum* with the aim to produce podophyllotoxin. Arens et al. (1986) studied a callus culture of *P.versipelle*, that accumulated flavonoids only. Except for these two studies, only one other report has been published dealing with the *in vitro* production of 5-methoxypodophyllotoxin by a root culture of *Linum flavum* L. (Berlin et al. 1986). Consequently, there was very little information available on the *in vitro* production of podophyllotoxin and related compounds at that stage. Recently, traces of authentic

podophyllotoxin have been found in undifferentiated cell suspensions of *L. flavum* (Wichers et al. 1991).

The main aim is the production of podophyllotoxin and related cytotoxic lignans using plant cell cultures. At the beginning of this study, plant species accumulating the highest levels of podophyllotoxin, *Podophyllum hexandrum* (Indian Podophyllum) and *Callitris drummondii* (Drummond's cypress pine), have been chosen for the establishment of plant cell cultures (Table 1). Unfortunately, it is known that both plants grow slowly and therefore difficulties might occur in obtaining well-growing cell cultures. In order to study the *in vitro* production of podophyllotoxin and related compounds in a broader context, callus and suspension cultures of *Linum flavum* (yellow flax), a plant with good growth and accumulating 5-methoxypodophyllotoxin in high quantities (Berlin et al. 1986), were initiated as well. The accumulation of podophyllotoxin and related compounds by the cell cultures derived from the three plant species is described in the Chapters 2, 3 and 4. In order to improve the production of the desired lignans, several approaches have been chosen and used throughout the thesis: manipulation with the growth medium (resulting in 'production media'), optimization of growth conditions, elicitation, bioconversion experiments (precursor feeding) and cell selection.

In Chapter 3, elicitation and precursor feeding experiments are described using cell suspensions of *L. flavum*. Chapter 5 describes the feeding of phenylpropanoids to cell suspensions of *P. hexandrum*. In Chapter 6, a new high-coniferin producing cell line of *L. flavum* is introduced. The relationship between this glucoside, lignan accumulation and the activities of two involved enzymes were investigated. In addition, an isolation procedure for coniferin is presented. Chapter 7 describes the initiation of a new selected root culture of *L. flavum* and the development of a production medium. Finally, in Chapter 8, the isolation of 5-methoxypodophyllotoxin from this root culture and its purification is described and its cytotoxic potency is compared with known cytotoxic compounds.

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CHAPTER 2

THE PRODUCTION OF PODOPHYLLOTOXIN BY CELL CULTURES DERIVED FROM *PODOPHYLLUM HEXANDRUM* ROYLE.

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ABSTRACT

The phenylpropanoid derived lignan podophyllotoxin, occurring in *Podophyllum* species, is used as a starting compound for the chemical synthesis of the anti-tumour agents etoposide (VP-16-213) and teniposide (VM-26). At present, the availability of this lignan becomes increasingly limited. As an alternative source, cell cultures originating from *Podophyllum hexandrum* Royle were initiated. Analysis of the cell extracts using TLC and different HPLC systems, indicated the presence of podophyllotoxin. After prepurification of the extracts by means of ITLC, the identity was confirmed by mass spectrometric analysis. Dark-grown cultures accumulated considerable higher amounts of podophyllotoxin in comparison with light-grown cultures.

INTRODUCTION

Podophyllum hexandrum Royle (Berberidaceae), also known as the Indian Podophyllum is a perennial herb, growing on the lower slopes of the Himalayas in scrub and forest from Afghanistan eastwards to central China (Chatterjee 1952). The rhizomes of *P. hexandrum* are known to contain several lignans (Jackson and Dewick 1984; Kamil and Dewick 1986; Tyler et al. 1988). Lignans are dimerization products of phenylpropanoid pathway intermediates linked by the central carbons of their side chain (MacRae and Towers 1984; Pelter 1986). The lignans occurring in *Podophyllum* possess anti-tumour properties, podophyllotoxin being the most active cytotoxic compound. The rhizomes of *P. hexandrum* contain ca. 4.3% podophyllotoxin on a dry weight basis (Jackson and Dewick 1984). Except for anti-tumour activities, also insecticidal and phytotoxic activities have been reported (MacRae and Towers 1984; Inamori et al. 1986). However, these lignans are too toxic for the treatment of neoplastic diseases in humans. Nevertheless, podophyllotoxin is used as the starting compound for the chemical synthesis of etoposide (VP-16-213) and teniposide (VM-26), both being applied successfully as anti-tumour agents. Their cytotoxic action is based on the inhibition of topoisomerase II, while podophyllotoxin is a spindle poison and acts as an inhibitor of the microtubule assembly (MacRae and Towers 1984; Richter et al. 1987; Holthuis 1988; Tyler et al. 1988; Van Maanen et al. 1988). These semi-synthetic analogues are used to treat a.o.: small cell lung

cancer, testicular cancer, neuroblastoma and hepatoma. The chemical structures of some lignans are shown in Chapter 1, Fig. 1.

The limited availability of the *P. hexandrum* plants, due to its long juvenile phase and poor fruit setting ability as well as the time-consuming collection of the plants results in a shortage of *Podophyllum* resin (podophyllin). Moreover, because of the non-optimal yields after extraction, podophyllotoxin is an expensive starting compound for the chemical synthesis of its derivatives (Chuang and Chang 1987). Therefore, the biotechnological production of podophyllotoxin using plant cell cultures derived from *P. hexandrum* has been considered to be an alternative. The occurrence of podophyllotoxin in callus cultures has only been demonstrated so far for *P. peltatum* (Kadkade 1981, 1982). In this chapter, the detection, identification and the accumulation of podophyllotoxin in undifferentiated callus and suspension-grown cells derived from *P. hexandrum* roots are described.

MATERIALS AND METHODS

PLANT MATERIAL

Podophyllum hexandrum Royle (synonym: *P. emodi* Wall.), Indian Podophyllum, belongs to the Berberidaceae, although the genus has sometimes been put into a separate family, the Podophyllaceae (Hutchinson 1959; Meacham 1980; Rix 1982). *Podophyllum* owes its name to the Greek *podos*, which means foot and *phyllon*, leaf, probably because of the shape of the leaves resembles a duck's foot. Rix (1982) reported on the existence of 16 different *Podophyllum* species although some are doubtfully distinct from each other. Plants occur in scrubs and forests from Afghanistan eastwards to central China, at heights of 2000-4500 m (Chatterjee 1952; Hutchinson 1959; Rix 1982; Dewick and Shaw 1988). Local names of *Podophyllum* include papra, papri, nirbash, bakra, bhavan-bakra (Chatterjee 1952). *P. hexandrum* is a herbaceous genus and has been in cultivation since 1820. It prefers to grow on dampish shady spots. *P. hexandrum* has a perennial rhizome, while the aerial parts are annual, emerging in the middle of April. The flowers appear in May, the fruit ripens in August or September (Chatterjee 1952). The plant bears 1 or 2 flowers, that open before the bronze/darkgreen leaves have unfurled. The white or pink flowers consist of 3 sepals, 6 petals, 2.5 to 4 cm long and 6 stamens. The 2 leaves are placed alternate on the flowering stem with petioles of 10-20 cm long. The leaves are circular and 12 to 25 cm across, deeply 3 to 5 lobed, toothed, often spotted and rugose. The fruit is the only part of the plant that is not toxic, and therefore edible but insipid (Chatterjee 1952; Hutchinson 1959; Rix 1982; Dewick and Shaw 1988). The chromosome number of *Podophyllum* has been determined to be 2n=12 (Meacham 1980).

Seeds of *P. hexandrum* Royle, were kindly provided by the Botanical Garden of Göttingen (Germany).

INITIATION OF TISSUE CULTURE AND CULTURE CONDITIONS

Seeds of *P. hexandrum* Royle, were surface sterilized by dipping into ethanol (70% v/v) for 1 min, followed by immersion for 30 min in a solution of sodium hypochlorite (3% w/v). Then the seeds were rinsed three times in sterile twice-deionised water and transferred to Perlite humidified with diluted (1:10) standard MS medium (Murashige and Skoog 1962) containing no phytohormones. Small roots developed after 1 to 3 months.

For the induction of callus from the explants of roots, a B5 medium (Gamborg et al. 1968) supplemented with 2% coconut milk, 4% sucrose, 4 mg l^{-1} naphthaleneacetic acid and solidified with 0.8% agar was used. The callus cultures were grown under a day/night regime (16/8 h; 3,000 lux) as well as in the dark and were transferred to fresh medium after a period of four weeks. Cell suspensions were initiated by transferring the callus to liquid medium with the same composition (*i.e.* without agar).
Cell suspension cultures of *P. hexandrum* were incubated on a rotary shaker (150 rpm) at 26 °C under a day/night regime (16/8 h; 3,000 lux) as well as in the dark and subcultured by adding 100 ml of a two-week-old cell suspension culture to 200 ml of fresh medium.

DETERMINATION OF GROWTH PARAMETERS

Suspension-grown cells were harvested during the growth cycle. Samples of ca. 10 ml were transferred to a calibrated conical tube followed by centrifugation for 5 min at 1,500 g. The medium pH and the conductance were routinely measured in the resulting supernatant. Cell dry weight (DW) was calculated from the weight difference of a glass fiber filter before and after suction filtration of an aliquot of cell suspension culture and subsequent drying at 60 °C for 24 h.

EXTRACTION PROCEDURE

The material resulting from the determination of the cell suspension dry weight or dried (24 h, 60 °C) five-week-old callus was powdered. Hundred mg of dry cell mass were extracted by ultra-sonification in methanol (80%; v/v) during 1 h. Dichloromethane (4.0 ml) and water (4.0 ml) were added and the mixture was vortexed. After centrifugation for 5 min at 1,500 g, two phases were obtained; 2.0 ml of the apolar dichloromethane phase were taken and evaporated to dryness, the residue was redissolved in 1.0 ml methanol and centrifuged for 2 min at 10,000 g (Eppendorf). For the determination of podophyllotoxin in the medium, 5.0 ml samples were extracted with 4.0 ml dichloromethane; 2.0 ml of the dichloromethane were evaporated to dryness and the residue taken up in 100 µl methanol. The resulting crude extracts were centrifuged for 2 min at 10,000 g (Eppendorf) and then submitted to the various methods of analysis.

THIN LAYER CHROMATOGRAPHY (TLC)

The crude extracts (30 µl) and podophyllotoxin (Sigma P-4405) as a reference compound (30 µl; 0.1 mg ml^{-1}) were chromatographed on silicagel 60-F-254 (Merck) plates using chloroform/methanol (9:1) over a distance of 10 cm in a saturated chamber. The spots were detected under UV light (254 nm) and developed with sulfuric acid/methanol (1:1), followed by heating during 10 min at 110 °C (Stahl 1970).

INSTANT THIN LAYER CHROMATOGRAPHY (ITLC)

The crude extracts (50 µl) and 30 µl podophyllotoxin (0.1 mg ml^{-1}) as a reference compound, were chromatographed on glass fiber impregnated with polysilicic acid gel and a fluorescent indicator (Gelman Instrument Company no. 51435). The conditions were as described under TLC., but here, only the reference zone was sprayed. The compound possessing the same R_f-value as the podophyllotoxin standard was isolated by cutting out the corresponding spot together with the stationary phase and redissolved in methanol by ultra-sonification during 15 min, followed by centrifugation for 5 min at 10,000 rpm (Eppendorf). The remaining methanolic fraction was evaporated to dryness.

MASS SPECTROMETRY

The compounds prepurified as described under ITLC were introduced into the mass spectrometer (Finnigan 3300) by means of direct sampling and identified by electron

impact (70 eV). Gas chromatography coupled with a mass spectrometer (GC-MS) was carried out with a Finnigan 4500 GC-MS-DS equipped with a fused silica column (12 m x 0.22 mm i.d.), while the mobile phase consisted of helium. The identification was by means of electron impact at 70 eV.
Podophyllotoxin, m/z, relative intensities (%): [M^+] 414(100%), 399(4%), 201(18%), 189(26%), 181(27%), 168(44%), 153(24%).

DETERMINATION OF PODOPHYLLOTOXIN GLUCOSIDES

To determine the presence of podophyllotoxin glucosides, the water phase of the methanolic extraction and the spent medium were submitted to enzymatic hydrolysis. A 3.5% (w/v) solution of β-glucosidase (Sigma G-0395) was prepared in 0.1 M phosphate buffer, pH 5.0. To 4.0 ml samples of the water phase or cell-free medium, 1.0 ml was added, followed by incubation during 5h at 37 °C. The aglucones formed were extracted as described under EXTRACTION PROCEDURE. The resulting extracts were analyzed by means of HPLC.

ANALYSIS BY MEANS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The standard HPLC system consisted of a Spectra-Physics (model SP 8810) liquid pressure pump, a Rheodyne high pressure valve equipped with a 10 µl sample loop and a Lichrosorb Si-60-7 (Chrompack) column (250 x 4.6 mm i.d.). The mobile phase used was n-heptane/dichloromethane/methanol (90:10:4). Analysis was performed at a flow rate of 2 ml min^{-1} (Lim and Ayres 1983). As a control on the retention behaviour of podophyllotoxin, a reversed phase Lichrosorb RP-18 (Chrompack) column (100 x 3 mm i.d.) was used, methanol/water (4:6) being the mobile phase at a flow rate of 1.2 ml min^{-1}. Podophyllotoxin was detected by means of UV absorbance at 290 nm using a Spectroflow (model 757) absorbance detector. Contents were calculated on authentic podophyllotoxin (Sigma P-4405). In order to determine the peak purity, podophyllotoxin containing extracts were analyzed by means of diode array detection (LKB 2140 Rapid Spectral Detector) at 230 and 286 nm, using the standard HPLC system. When the logarithm of the absorbance 230 nm/ absorbance 286 nm ratio has a constant value, during peak appearance, 100% peak purity is measured.

CALCULATION OF PRODUCTION RATE

The production rate is defined as the difference of the maximal yield and the yield at the time of inoculation, divided by the corresponding period of time, and is expressed as mg product per liter per day (mg l^{-1} day^{-1}).

RESULTS AND DISCUSSION

Callus cultures of *Podophyllum hexandrum* Royle were very difficult to initiate. It appeared to be impossible to obtain sterile root or rhizome explants from *in vivo* collected plant material or to induce callus formation on stem and leaf pieces, under the conditions chosen. Therefore, *in vitro* plantlets were used as starting material for the induction of callus cultures. Furthermore, several hundreds of medium compositions were tested, including variation in the basal medium composition, hormone combinations and concentrations, carbon sources, complex supplements such as coconut milk and casamino acids as well as illumination conditions. Finally, the medium composition and the culture

conditions as described under MATERIALS AND METHODS, gave satisfactory results in terms of cell growth. From this callus, homogeneous and undifferentiated suspensions were obtained. After 10 passages, their dry weight ranged from approximately 15 to 30 g l^{-1} during one growth cycle. Cultures belonging to one cell line sometimes tended to change colour spontaneously. Good podophyllotoxin producing cell cultures are dark-brown coloured. When the colour changed to yellow/green, this was accompanied by the complete loss of the podophyllotoxin production. Therefore, only dark-brown calli and cell suspensions were used in this study.

Normal phase HPLC analysis of the crude extracts of *P. hexandrum* cultures yielded remarkable simple chromatograms, only three well-separated peaks were visible. The retention time of podophyllotoxin under the conditions applied was ca. 19 min. A first control of this analysis was done by using the reversed phase HPLC column, confirming the presence of a compound having the same retention time as authentic podophyllotoxin. When the extracts were spiked with authentic podophyllotoxin, using both HPLC systems, no additional peak appeared in the HPLC-chromatogram, being a stronger indication for the presence of podophyllotoxin. By performing HPLC in combination with diode array detection, it was confirmed that the peak 'consisted' of pure podophyllotoxin. (see Fig. 1).

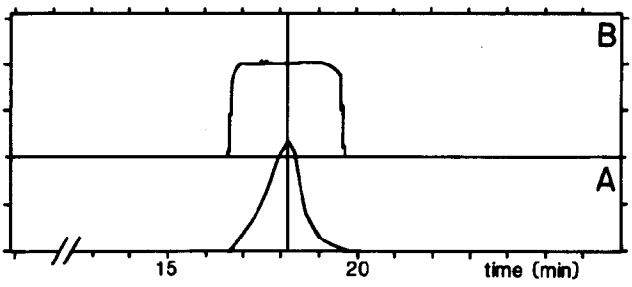

Fig. 1. Diode array detected HPLC chromatogram of a *P. hexandrum* cell extract which is spiked with authentic podophyllotoxin. **A.** Absorbance at 286 nm. **B.** Log (Absorbance 230 nm/ Absorbance 286 nm).

Analysis by means of TLC gave characteristic dark blue spots (R_f-value 0.55) made visible by spraying with sulfuric acid/methanol. Further purification of the extracts through the method of ITLC, proved to be an easy way to isolate small amounts of podophyllotoxin. The identity of the isolated compound was confirmed by mass spectrometry based on the characteristic fragmentation pattern (Duffield 1967). Gas chromatography coupled with mass spectrometry (GC-MS) carried out with crude extracts indicated that also traces of the lignans β-peltatin and desoxypodophyllotoxin (see

Chapter 1, Fig. 1 and Fig. 4, respectively) were present, contents being less than 0.001% on a dry weight basis.

The undifferentiated five-week-old callus cultures of *P. hexandrum* are able to accumulate podophyllotoxin, concentrations usually ranging from 0 to 0.10% on a dry weight basis. The average content was 0.017 ± 0.042% (n=25). The highest content of podophyllotoxin measured, was in dark-grown callus, 0.30% podophyllotoxin on a dry weight basis. In general, callus cultures contained the same levels of podophyllotoxin in comparison with suspension-grown cells.

Cell suspensions grown in the light contained 3-4 times less amounts of podophyllotoxin when compared with dark-grown cultures, while the dry weight increase was nearly the same.

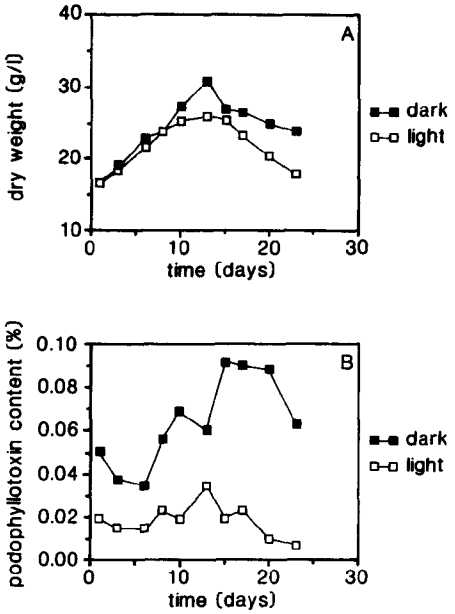

Fig. 2. Characteristics of growth (**A**) and podophyllotoxin accumulation (**B**) of a suspension culture of *P. hexandrum*, grown under dark and light conditions.

Growth characteristics in terms of dry weight changes and the podophyllotoxin accumulation in dark- as well as light-grown suspension cultures of *P. hexandrum* are shown in Fig. 2. The production of podophyllotoxin started at day 5 after a slight decrease in the first part of the growth cycle and generally corresponded to changes in dry weight. The highest cellular accumulation of podophyllotoxin in suspension-grown cells was measured at day 15, during the stationary phase of the growth cycle, followed by a decrease in podophyllotoxin content. Only negligible amounts of podophyllotoxin were

detected in the medium. No glucosides of podophyllotoxin in cells or medium could be detected. The production rates calculated for the light- and dark-grown cultures were 0.5 (13 days) and 1.3 mg (15 days) podophyllotoxin l^{-1} day^{-1}, respectively.

In strong contrast with our results are those reported for the podophyllotoxin production in cultures derived from the rhizomes of *P. peltatum*, which was stimulated upon illumination (Kadkade 1981, 1982). Apparently, illumination has no direct influence on the podophyllotoxin production. Obviously, the choice of *Podophyllum* species and culture conditions, as well as other complex factors are involved in the biosynthesis of podophyllotoxin.

Judging from the results presented in this paper, it may be concluded that undifferentiated plant cells derived from *P. hexandrum* roots are able to produce podophyllotoxin endogenously. So, in principle the biotechnological production of podophyllotoxin *in vitro* is possible and can be improved further (Chapter 5).

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CHAPTER 3

THE PRODUCTION OF 5-METHOXYPODOPHYLLOTOXIN BY CELL SUSPENSION CULTURES DERIVED FROM *LINUM FLAVUM* L.

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ABSTRACT

Cell suspension cultures of *Linum flavum* L., routinely grown on a NAA-containing medium, accumulated low levels of the phenylpropanoid-derived lignan 5-methoxypodophyllotoxin (5-MPT), up to 0.015% on a dry weight basis. Feeding experiments with the precursor L-phenylalanine resulted in a 3-5 fold increase in 5-MPT levels, but caused the levels of phenylalanine ammonia-lyase (PAL; E.C.4.3.1.5) activity to fall. Treatment of the cultures with the elicitor Nigeran, either alone or in combination with phenylalanine, caused the 5-MPT production to cease, even though PAL activity was rapidly enhanced by these treatments. Transfer of the cultures to NAA-free medium resulted in a 40-50 fold higher level of 5-MPT accumulation, the PAL activity levels being lowered compared with the routinely grown cells. With these more differentiated cultures, L-phenylalanine-feeding had no effect on the 5-MPT content, while elicitor treatment, both on its own and in combination with the precursor, caused the 5-MPT production to cease again. Under all these experimental conditions using NAA-lacking cell cultures, the PAL activities were higher than that of the untreated culture. No general correlation could be found between PAL activities and 5-MPT contents.

INTRODUCTION

Some plant species like *Podophyllum* (Kadkade 1982; Fay and Ziegler 1985) and *Linum* (Weiss et al. 1975; Berlin et al. 1986, 1988) synthesize podophyllotoxin and/or related compounds. These lignans are derived from the phenylpropanoid pathway. Their synthesis involves dimerisation of phenylpropanoid units (Jackson and Dewick 1984; Pelter 1986). Lignans possess interesting biological activities, their cytostatic properties being of most importance. The chemically prepared derivatives of podophyllotoxin, etoposide (VP-16-213) and teniposide (VM-26), are well-known anti-tumour agents. Their action is based on the inhibition of topoisomerase II (Richter et al. 1987; Beers et al. 1988; Holthuis 1988), and they are used for the treatment of small lung cell cancer, testicular cancer neuroblastoma and hepatoma (Holthuis 1988). Next to their anti-tumour activities, podophyllotoxin and many related compounds possess insecticidal, phytotoxic and anti-viral activities (Bedows and Hatfield 1982; Inamori et al. 1986). The structural formulas of some lignans and derivatives are shown in Chapter 1, Fig. 1.

Recently, it has been reported that root cultures of *Linum flavum* L. are able to produce large amounts (0.7-1.3% on a dry weight basis) of the closely podophyllotoxin related lignan 5-methoxypodophyllotoxin (5-MPT) (Berlin et al. 1986, 1988). The chemical structure is depicted in Fig. 1. From our undifferentiated cell lines derived from this plant we have isolated (0.004-0.015 % on a dry weight basis) and identified 5-MPT.

Plant cells respond to elicitation by microbe-derived cell wall components (elicitors) by synthesizing a complex mixture of defence-related molecules termed phytoalexins, some of which are produced by the phenylpropanoid pathway, as is 5-MPT (Dixon et al. 1979; DiCosmo and Misawa 1985; Dixon 1986; Ebel 1986; Funk et al. 1987; Imoto and Ohta 1988). Phenylalanine ammonia-lyase (PAL) is a key enzyme in phenylpropanoid biosynthesis and converts L-phenylalanine into *trans*-cinnamic acid.

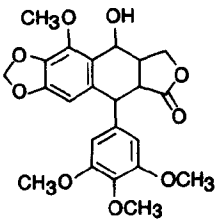

Fig. 1. Chemical structure of 5-methoxypodophyllotoxin.

PAL is sensitive to elicitation by biological and physical agents (Dixon et al. 1979; Gerrish et al. 1985; Rolfs et al. 1987). Treatment of parsley (*Petroselinum hortense*) cultures with an elicitor derived from either *Phytophtora megasperma* or *Alternaria carthami* results in a rapid induction of PAL and other enzymes from the general phenylpropanoid pathway (Tietjen and Matern 1983).

The main goal of this study was to improve the production of 5-MPT by cell cultures of *L. flavum* and to investigate the relationship between 5-MPT accumulation and PAL activity. Phytohormones can affect the production of secondary metabolism in plant cell cultures (Knobloch and Berlin 1980; Kadkade 1982; Kurz and Constabel 1985; Misawa 1985; Berlin 1988) Suspension cultures of *L. flavum*, routinely grown on a NAA-containing medium, were transferred to a hormone-free medium, and the PAL activity as well as the 5-MPT content of the cultures were measured. In addition, L-phenylalanine was added as a precursor to cell cultures grown on NAA-containing and hormone-free medium. To investigate whether the production of 5-MPT can be increased by means of elicitation, Nigeran (a commercially available polyglucan isolated from *Aspergillus japonicus*) was added as a non-specific elicitor to the cell cultures, both on its own and in combination with L-phenylalanine.

MATERIALS AND METHODS

PLANT MATERIAL

Linum flavum L. (Linaceae), yellow or golden flax, is a member of the section *Syllinum* of this genus and is a native of Southern, Central and Eastern Europe (Tutin 1968; Everest 1981). The flax family contains 230 species, whereas the section *Syllinum* consists of 12 distinct species (Tutin 1968). The yellow flax generally attains a height of about 60 cm and has stems that become slightly woody at the bases. The variety *L. flavum* 'Compactum' is shorter.
L. flavum leaves are alternate, the lower leaves are bluntly-obovate, those above are narrower and more pointed. The leaves are approx. 5 cm in length, lanced, sessile, stout, smooth and glaucous. At each side of the base of the leaves there is a gland. The perennial and herbaceous plants bear abundunt gold-yellow flowers that are 2.5 cm in diameter, are produced in branched heads and in numerous clusters. The flowering period is August-September. The 5 sepals of 6-8 mm are lanceolate and usually glandular-ciliate. The petals are approx. 20 mm with a relatively short claw. The capsule that contains the 10 flat seeds is globular with a beak of 1-2.5 mm (Wood 1898; Tutin 1968; Everest 1981).
Seeds of *L. flavum* L. (Linaceae), from plants growing at the Hortus Botanicus of the University of Utrecht, were provided by Dr. H.J. Wichers from TNO-ITC, Zeist.

INITIATION OF TISSUE CULTURE AND CULTURE CONDITIONS

Seeds of *Linum flavum* L. (Linaceae), were sterilized by dipping into 70% (v/v) ethanol for 1 min followed by immersion for 15 min in a solution of sodium hypochlorite (3%; w/v). The seeds were rinsed three times in sterile, twice-deionised, water and transferred to a medium containing MS salts (Murashige and Skoog 1962) and B5 vitamins (Gamborg et al. 1968), supplemented with 2% sucrose, 0.88 mg l^{-1} folic acid, 2 mg l^{-1} glycine and solidified with 0.8% agar. Plantlets were obtained after 3 weeks, and the leaves were used for callus induction on the medium described above, but now 3% sucrose and 3 mg l^{-1} NAA were added (standard medium). After 2 months of subculturing, cell suspensions were initiated by transferring the callus to the standard liquid medium (*i.e.* without agar). The suspension cultures were incubated on a rotary shaker (150 rpm) at 26 °C under a day/night regime (16/8 h; 3,000 lux) and subcultured by adding 100 ml of a two-week-old cell suspension culture to 200 ml of fresh medium.

ELICITOR EXPERIMENTS

Nigeran (Sigma no. N-2888), a polyglucan isolated from the fungus *Aspergillus japonicus* was used as an elicitor. A 5.0 mg ml^{-1} suspension of Nigeran in twice-deionised water was prepared. After autoclaving, the clear solution was cooled under ultra-sonification, resulting in a homogeneous suspension. For the inoculation of the cell suspension cultures, 75 ml of cells of a two week old suspension were added to 300 ml of fresh medium. To these cultures, 12.0 ml of the Nigeran suspension was added at day 8 of the growth cycle, resulting in an elicitor concentration of 0.2 g l^{-1}, *i.e.* approx. 14 μg mg^{-1} dry weight. To the control cultures 12.0 ml of twice-deionised water was added. The same procedure was followed with a medium containing 3.0 mM L-phenylalanine as a substrate. In addition, all these experiments were carried out using a NAA-lacking medium.

DETERMINATION OF GROWTH PARAMETERS

Cells were harvested during the growth cycle. The packed cell volume (PCV) was determined after transfer of ca. 10 ml cell suspension to a calibrated conical tube followed by centrifugation for 5 min at 1,500 g. The medium pH and conductance were routinely measured in the resulting supernatant. Cell dry weight (DW) was calculated from the weight difference of a glass fiber filter before and after suction filtration of an aliquot of cell suspension culture, and subsequent drying at 60 °C for 24 h.

EXTRACTION PROCEDURE

5-MPT was extracted according to the procedure that has been described previously for podophyllotoxin (Van Uden et al. 1989; Chapter 2).

DETERMINATION OF 5-MPT-β-D-GLUCOSIDE

5-MPT-β-D-glucoside was determined by following the procedure as described for podophyllotoxin-β-D-glucoside (Van Uden et al. 1989; Chapter 2)

ANALYSIS BY MEANS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The HPLC system consisted of a Spectra-Physics (model SP 8810) liquid high pressure pump, a Rheodyne high pressure valve equipped with a 10 µl sample loop and a Lichrosorb Si-60-7 (Chrompack) column (250 x 4.6 mm i.d.).
The mobile phase was n-heptane/dichloromethane/methanol (90:10:4) (Lim and Ayres 1983). Analysis was performed at a flow rate of 2 ml min^{-1}. 5-MPT was detected by means of UV absorbance at 290 nm using an Analytical Kratos Division (model Spectroflow 757) absorbance detector. An impure sample of 5-MPT, prepared in our laboratory, according to the ITLC procedure used for the isolation of podophyllotoxin from cells of *Podophyllum hexandrum* (Van Uden et al. 1989), was used for the determination of the retention time. Since no 5-MPT as a reference was available, relative contents were initially calculated on pure podophyllotoxin (Sigma no. P-4405). Later, when 5-MPT was isolated and purified, the contents were recalculated. The production rates were calculated as described in Chapter 2.

PHENYLALANINE AMMONIA LYASE (PAL; E.C.4.3.1.5) ACTIVITY

Cells of *L. flavum* suspension cultures (ca. 0.5 g fresh weight) were ground in liquid nitrogen and transferred to 2.0 ml 0.1 M sodium borate buffer (pH 8.8) containing ca. 100 mg Dowex (Sigma no. 1x2-100) in order to remove phenols. The extract was centrifuged at 17,000 g for 10 min at 4 °C. The PAL activity was determined spectrophotometrically according to the method of Zucker (1965) and is expressed as µkat kg^{-1} protein.

PROTEIN CONTENT

Protein was determined according to the method of Bradford (1976) using bovine serum albumin (BSA, Sigma, no. A 7638) as the standard protein.

RESULTS AND DISCUSSION

CULTURE CHARACTERISTICS

In cells of *Linum flavum* grown on standard medium, 5-MPT was accumulated intracellularly, although during the latter part of the stationary phase, negligible amounts of product were detected in the medium. No 5-MPT-β-D-glucoside could be detected in cells or medium. From Fig. 2 it can be seen that the 5-MPT content of the cells was highest in the stationary phase of the growth cycle, *i.e.* 0.015%, calculated on a dry weight basis.

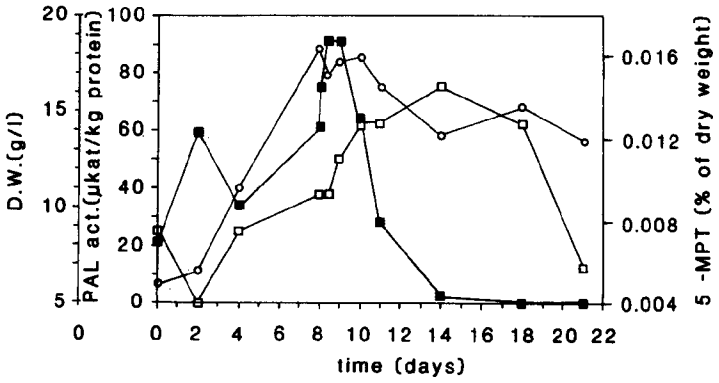

Fig. 2. Time course of the changes in cell dry weight (o), activities of PAL (■) and the intracellular 5-MPT accumulation of standard-grown cell cultures of *L. flavum* (□).

It had been demonstrated that the elicitor-treatment of cell cultures is most effective, when the content of secondary metabolites is at its maximum (Funk et al. 1987; Rolfs et al. 1987; Schumacher et al. 1987). Therefore, in this study, the elicitor (Nigeran) was added at the beginning of the stationary phase of the growth cycle. The dry weight of routinely grown cell cultures increased from 6 g l^{-1} to 18 g l^{-1} during the first 8 days of cell culture. No decrease in cell dry weight was observed, after the omittance of NAA or after addition of the elicitor. The medium conductance decreased from 4.4 mS at day 1 to ca 1.7 mS at day 21 under all experimental conditions. The PAL activity reached a maximum value of 92 µkat kg^{-1} protein at day 9 of the growth cycle, *i.e.* 5 days before the highest 5-MPT accumulation was reached.

5-MPT PRODUCTION

Cells of *L. flavum* grown under standard conditions accumulated only low levels of 5-MPT, 0.015% on a dry weight basis and this corresponded with a production rate of 0.1

mg l^{-1} day^{-1} (14 days). When these cells were transferred to a hormone-free medium, the cells immediately started accumulating 5-MPT (Fig. 3). The 5-MPT content of the plant cells reached levels of 0.58-0.73%, *i.e.* a 40-50 fold increase of product formation at day 18 of the growth cycle, the production rate was 4.8 to 6.0 mg l^{-1} day^{-1} (18 days). This is probably a direct result of cell-differentiation, as some differentiation occurred. Although the undifferentiated cultures have been maintained for over two years, this rapid redifferentiation is quite remarkable.

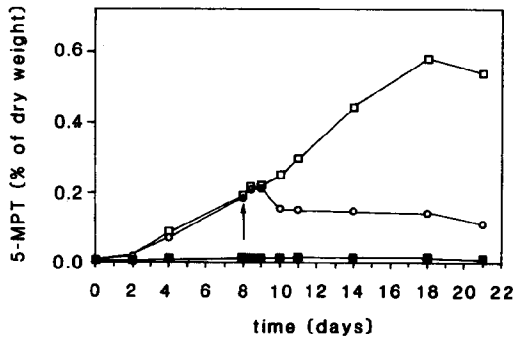

Fig. 3. The intracellular accumulation of 5-MPT, in standard-grown (■), and in elicitor treated (o) and untreated (□) cells of *L. flavum* grown on NAA-free medium. The arrow indicates the time of elicitor addition.

On adding L-phenylalanine to the cell suspensions grown under standard conditions, the 5-MPT production could be increased 3-5 fold (Table 1), the production rates were 0.4-0.6 mg l^{-1} day^{-1}. However, no stimulation of 5-MPT production was observed when the L-phenylalanine was present in the hormone-free medium. The incorporation of L-phenylalanine into podophyllotoxin had already been demonstrated with plants of *Podophyllum hexandrum* Royle (Ayres 1981; Ayres et al. 1981). The above results suggest that it can be incorporated into 5-MPT by freely suspended cells of *L. flavum*.
The effect of the added elicitor on the accumulation of 5-MPT was rather dramatic, since the intracellular accumulation of this lignan ceased. (Fig. 3) For the standard-grown cells, the effect of the elicitor is not shown in this figure, since the 5-MPT contents were in the range 0-0.007% on a dry weight basis. The underlying mechanism for this observation is not clear. In contrast to elicitor-treated cell suspensions of *Solanum melongena*, *Petroselinum hortense* and *Nicotiana tabacum*, where release of secondary metabolites into the medium occurred (Tietjen et al. 1983; Chapell and Nable 1987; Imoto and Ohta 1988), no release of 5-MPT was observed for cultures of *L. flavum*.

PAL ACTIVITY

In general, cell suspensions of *L. flavum* showed an increase in PAL activity 2 days after transfer to fresh medium. Such a peak of enzyme activity shortly after transfer has been

observed before (Rolfs et al. 1987). The PAL activity in cell suspensions, which were not treated with elicitor, was maximal at day 9-10 of the growth cycle. During the stationary phase, the PAL activity decreased to a very low level; values of 0-5 μkat kg^{-1} protein were measured (Fig. 2-6). The transfer of the routinely grown cultures to the NAA-free medium, resulted in more differentiated plant cells, possessing lower levels of PAL activity (Fig. 4).

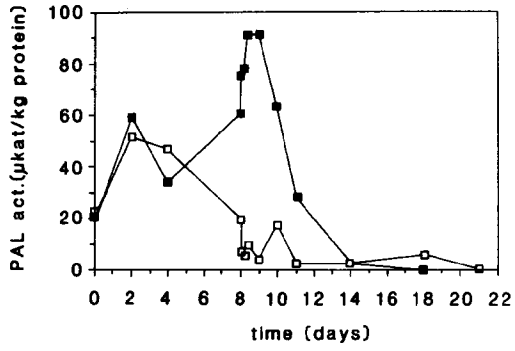

Fig. 4. Time course of the PAL activity levels in cell suspensions of *L. flavum*, grown in NAA-containing (■) and NAA-free (□) medium.

The feeding of L-phenylalanine to routinely grown cells, gave a ca. 7-fold decrease of PAL activities, while cells grown on the NAA-free medium, by contrast, had a ca. 2 fold higher level of PAL activity, both compared to the PAL activity of cells growing without L-phenylalanine (Table 1). This decrease of PAL activity for the standard-grown cells is depicted in Fig. 5.

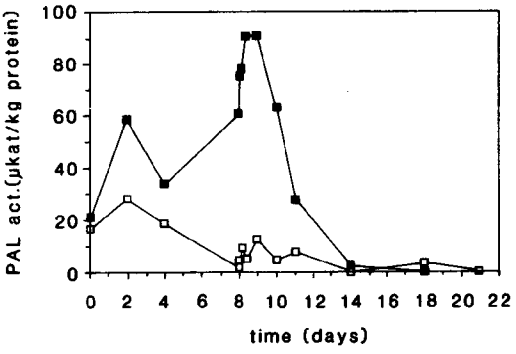

Fig. 5. Time course of the PAL activity levels in cell suspensions of *L. flavum*, grown in L-phenylalanine-containing (□) and L-phenylalanine-free (■) standard medium.

The inhibitory effect of L-phenylalanine on PAL activity levels is not uncommon. Zucker (1965) showed that potato disks cultured on L-phenylalanine in either light or dark contained less than half as much enzyme activity as corresponding disks supplied only

with water. A similar effect was reported by Margna (1977), who administered L-phenylalanine to seedling tissues of buckwheat and radish, and observed a decrease in PAL activities. At present, this precursor inhibition effect is not well understood.

The addition of Nigeran as an elicitor to the routinely grown cells, as well as to those grown on NAA-free medium, resulted in a rapid response in terms of changes in PAL activity. Generally, the PAL activity was increased within 2-5 hours, reaching maximal values at day 9-10, *i.e.* 24-48 hours after elicitor addition. This is shown in Fig. 6 for the standard-grown cells.

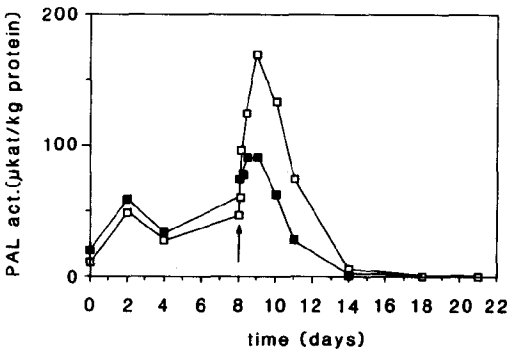

Fig. 6. The PAL activity levels of an elicitor-treated (□) and -untreated (■) cell suspension of *L. flavum*, grown under standard conditions. The arrow indicates the time of elicitor addition.

The highest PAL activity was measured, after elicitor treatment, when a value of 170 μkat kg^{-1} protein was found (Table 1). A 2-5 fold increase of the PAL activity was observed upon elicitor treatment, except for the combination with L-phenylalanine. These results are in good agreement with those reported for the PAL activities in elicitor-treated cell suspensions of *Arachis hypogaea* and *Glycine max* (Funk et al. 1987; Rolfs et al. 1987). Administration of Nigeran in combination with L-phenylalanine to cultures grown on the standard medium gave a decrease of PAL activity levels; cells grown on the NAA-free medium, on the contrary, gave an increase in these levels (Table 1). Apparently, more differentiated cells respond in a different way to elicitor-treatment and precursor-feeding.

RELATIONSHIP BETWEEN PAL-ACTIVITY AND 5-MPT PRODUCTION

In order to study the relationship between PAL activity and 5-MPT accumulation, the highest activities, measured between day 8-11, as well as the maximum 5-MPT contents of the *L. flavum* suspension cultures under the various experimental conditions, are summarized in Table 1. From these results, it can be seen that a high activity of PAL does not lead to a high 5-MPT accumulation. A lack of correlation between changes in the level of PAL and the accumulation of phenylpropanoids has been observed previously (Camm

1973; Margna 1977), although mostly positive correlations have been found (Dixon and Lamb 1979; Funk et al. 1987; Rolfs et al. 1987; Schumacher et al. 1987).

Table 1. Highest levels of PAL activity (between 8 and 11 days) and maximal 5-MPT contents observed in cell suspensions of *L. flavum* grown under a variety of experimental conditions.

Conditions	PAL (μkat kg^{-1} protein)		5-MPT (% DW)	
	+ NAA	- NAA	+ NAA	- NAA
standard	92	17	0.015	0.58
+ elicitor treatment	170	97	0.007	0.21
+ 3 mM L-phenylalanine	13	32	0.047	0.58
+ 3 mM L-phenylalanine + elicitor	41	40	0.011	0.26

An explanation for our findings may be that there are sufficient, non-limiting levels of PAL activity present. Thus, an enhancement of this activity need not to be followed by an equivalent increase of phenylpropanoid derived products; the enzyme activity is not the limiting factor, but possibly the substrate supply. In our investigations, this hypothesis was supported by the enhancement of the 5-MPT production in cells grown on NAA-containing medium, after addition of the precursor L-phenylalanine.

PAL is a key enzyme in the early part of the the phenylpropanoid pathway (Ebel 1986). It was not surprising, that in the undifferentiated as well as in the more differentiated cultures of *L. flavum*, PAL activity was nearly always detectable under all experimental conditions. No general correlation could be found between PAL activities and the 5-MPT production.

FINAL CONCLUSIONS

From the results of this study, some interesting conclusions may be drawn. Enhancement of PAL activity by means of elicitation did not lead to an increase in 5-MPT accumulation. L-phenylalanine is a precursor of 5-MPT and when added to standard-grown cultures, a 3-5 fold increase of 5-MPT accumulation was measured. A far more efficient method to increase 5-MPT production is to transfer standard-grown cultures to a phytohormone-free medium. This results in a 40-50 fold higher 5-MPT content. To our knowledge, no such large increase of accumulation has been reported before, by simply transferring plant cell suspension cultures to a medium which does not contain phytohormones. The transfer of *L. flavum* cell suspensions to our production medium leads to 5-MPT contents of 0.58-0.73%, on a dry weight basis. The highest production rate calculated was 6.0 mg 5-MPT l^{-1} day^{-1}. These results offer good prospects for the improvement of the production using differentiated cell cultures of *L. flavum*.

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CHAPTER 4

THE PRODUCTION OF PODOPHYLLOTOXIN-β-D-GLUCOSIDE BY CELL SUSPENSION CULTURES DERIVED FROM THE CONIFER *CALLITRIS DRUMMONDII* F. MUELLER.

(Published in Plant Cell Reports 9: 257-260)

ABSTRACT

Podophyllotoxin was produced by cell cultures derived from the needles of *Callitris drummondii*. The needles of this conifer contained 1.56% podophyllotoxin on a dry weight basis, of which 32% was present in its β-glucosidic form. Trace amounts of desoxypodophyllotoxin and matairesinol were detected as well. In dark-grown cell cultures ca. 0.02% podophyllotoxin was accumulated, 85-90% in the β-D-glucosidic form. Moreover, traces of the lignans matairesinol, hinokinin and asarinin were found. Illumination stimulated the endogenous production of podophyllotoxin-β-D-glucoside; contents up to 0.11% could be measured.

INTRODUCTION

It has been recognized for a long time that conifer species, such as *Libocedrus*, *Podocarpus*, *Callitris* and *Juniperus*, contain compounds with tumour-damaging capacities. These properties were ascribed to: savinin, matairesinol, hinokinin, desoxypodophyllotoxin and podophyllotoxin. All these compounds belong to the lignans (Fitzgerald et al. 1953, 1957; Erdtman 1955).

Podophyllotoxin-β-D-glucoside (Fig. 1) has been isolated for the first time from the needles of *C. drummondii* (Kier et al. 1963). The podophyllotoxin content was 1.4% on a dry weight basis.

The possibility to produce podophyllotoxin by means of undifferentiated plant cell cultures has already been shown by Kadkade (1981, 1982) for *Podophyllum peltatum* as well as by Van Uden et al. for *Podophyllum hexandrum* (1989; Chapter 2).

It has also been reported that plant cell cultures of *Linum flavum* produce a closely related lignan, 5-methoxypodophyllotoxin (Berlin et al. 1986 and 1988; Van Uden et al. 1990; Chapter 3). In the current study, the detection and identification of podophyllotoxin and its β-D-glucoside, accumulated in cell cultures of the conifer *C. drummondii* are presented, and the contents found are compared with those in the needles of the intact plant.

MATERIALS AND METHODS

PLANT MATERIAL

Callitris drummondii (Parlatore) F. Mueller, vernacularly named Drummond's Cypress pine, belongs to the family of Cupressaceae (Dallimore and Jackson 1966). Appreciable nomenclature complexities occur and the number of *Callitris* species as mentioned in various reports lies between 13 and 20 (Li 1953; Dallimore and Jackson 1966; Gadek and Quinn 1983; Adams and Simmons 1987; Evans 1989). The name *Callitris* is derived from the Greek word *kallistos* that means most beautiful. *C. drummondii* is a native 17 m high shrub or tree of the coast of West Australia. *Callitris* species are very well adapted for dry and arid regions. The bark is hard and furrowed. The branches are angular and divide into slender branchlets which are ridged by the closely pressed, sheath-like bases. The buds are hidden by the needles which are longer than in most other species and are pressed close to the branch except at the blunt tip. The male and female strobili occur on the same plant. Each cone contains ca. 6 oblong seeds which have 1 broad wing. The cones are placed solitary or in clusters, appear on stout stalks and are almost spherical with a diameter of ca. 1 cm. The scales are thick and woody, alternate scales only slightly smaller than the others (Dallimore and Jackson 1966). A detailed description of cone and ovule development has been given by Takaso and Tomlinson (1989).
The needles used in this study originated from *Callitris drummondii* (Parlatore) F. Mueller (Cupressaceae). The plant was obtained from the Pinetum Blijdenstein in Hilversum, The Netherlands and its identity was confirmed according to the description of Dallimore and Jackson (1966).

INITIATION OF TISSUE CULTURE AND CULTURE CONDITIONS

The needles of a ca. three-months-old plantlet of *C. drummondii*, were surface sterilized by 10 min immersion in a 1% (w/v) solution of sodium hypochlorite. Then the needles were rinsed three times in double-deionised water and cut into small pieces. In order to initiate callus growth, these needle explantates were transferred to a B5 medium (Gamborg et al. 1968) or an MS medium (Murashige and Skoog 1962), both media contained 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid as a phytohormone and were solidified with 0.8% (w/v) agarose (Sigma) or agar (Difco). The obtained callus cultures were grown under a day/night regime (16/8 h; 3,000 lux). Cell suspensions were initiated by transferring 4- to 8-week-old calli to a B5 medium, which was supplemented with 4% (w/v) sucrose and 4 mg l^{-1} naphthaleneacetic acid. The cell suspensions were incubated on a rotary shaker, 150 rpm, at 26 °C either under a day/night regime (16/8 h; 3,000 lux) or in continuous dark. Subculturing was done by adding 150 ml of fresh medium to 150 ml of a 3-week-old suspension culture.
Culture characteristics were determined as described previously for *Podophyllum hexandrum* (Van Uden et al. 1989; Chapter 2).

EXTRACTION PROCEDURE

Crude extracts to be used for the various methods of analysis were prepared according to Van Uden et al. (1989; Chapter 2).

DETERMINATION OF PODOPHYLLOTOXIN GLUCOSIDES

Podophyllotoxin and podophyllotoxin-β-D-glucoside were quantified according to Van Uden et al. (1989; Chapter 2).

METHODS OF ANALYSIS

All methods of analysis, *i.e.* HPLC-UV, TLC, Instant TLC (ITLC), and mass spectrometry were used as described previously (Van Uden et al. 1989; Chapter 2). The production rates were calculated as reported in Chapter 2.
Mass spectral data of lignans, occurring in extracts from the needles and cell suspensions of *C. drummonddi*, as detected with GC-MS are, m/z, relative intensities (%): podophyllotoxin [M^+] 414(38), 399(5), 201(38), 189(38), 181(47), 168(100); desoxypodophyllotoxin [M^+] 398(100), 230(19), 185(42), 181(66), 173(55), 168(25); matairesinol [M+] 358(14), 221(14), 137(100); asarinin [M^+] 354(18), 323(3), 203(18), 178(5), 161(37), 149(100), 135(61); hinokinin [M^+] 354(11), 219(3), 192(4), 162(8), 135(100), 77(25).

RESULTS AND DISCUSSION

Primary callus cultures of *Callitris drummondii* were readily obtained. Already after ca. two weeks the first callus tissue was visible. However, we did not succeed in maintaining well-growing callus cultures of *C. drummondii*. After 6-8 weeks, the colour of the primary callus turned from green to dark-brown, while no further growth was observed. Attempts to improve the conditions for callus growth were made by replacing the agar by agarose or by putting sterile, moistened paper on the agar surface, in order to avoid direct contact between agar and plant cells. Particularly, this contact between cells and agar seemed to be disadvantageous. When a well-growing cell suspension of *C. drummondii* was subcultured by adding fresh medium, which was supplemented with 0.1% agar, cell growth ceased immediately. With respect to callus growth, the best results were obtained using agarose to solidify the medium. For the initiation of cell suspension cultures of *C. drummondii* it was necessary to use 4- to 8-week-old calli. In contrast with the callus cultures, the maintenance of the cell suspensions gave no problems, and these cultures have been growing stably, though slowly, for over 4 years in our laboratory.
The results of various methods of analysis (TLC, HPLC) indicated the presence of podophyllotoxin in callus and suspension material. Purification of the extracts by ITLC, proved to be a simple way to isolate podophyllotoxin. Its identity was confirmed by direct sampling mass spectrometry. In this way the characteristic fragmentation pattern of podophyllotoxin (Duffield 1967) was obtained. In order to detect trace amounts of related lignans, possibly present in the samples, crude extracts of needles and of cell suspension material of *C. drummondii* were subjected to gas chromatography coupled with mass spectrometry (GC-MS). The mass spectra revealed clearly the presence of podophyllotoxin in all samples. Furthermore, traces of other lignans were found, namely desoxypodophyllotoxin and matairesinol in needle material, and matairesinol, hinokinin and asarinin in cell suspension material. Our mass spectrometric data were in agreement with those reported previously for these lignans (Duffield 1967; Dewick and Jackson

1981). These lignans could not be quantified by HPLC, as no reference compounds are available. The occurrence of these closely related lignans (see Fig. 1) in our tissue culture material and needles was to be expected.

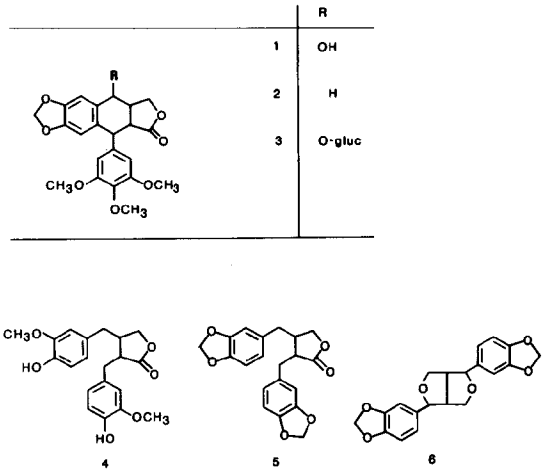

Fig. 1. Chemical structures of lignans occurring in *Callitris drummondii* needles and derived cell cultures. Podophyllotoxin (**1**), desoxypodophyllotoxin (**2**), podophyllotoxin-β-D-glucoside (**3**), matairesinol (**4**), hinokinin (**5**) and asarinin (**6**).

The presence of matairesinol and hinokinin has already been described for several other conifer species. (Fitzgerald 1953; Krahmer et al. 1970; Shain and Hillis 1971; Matsubara 1972). Matairesinol has been proposed to be a branchpoint intermediate between the trimethoxy- and the dimethoxy series of the aryltetralin lignans in *Podophyllum* plant species (Kamil and Dewick 1986). However, matairesinol has not been detected so far in the high-lignan containing rhizomes of *Podophyllum* species.

Determination of the podophyllotoxin content in the needles of *C. drummondii*, yielded 1.56% on a dry weight basis, of which ca. 25% was present in the β-D-glucosidic form. The aglucone was readily obtained after enzymatic hydrolysis by a β-glucosidase (E.C. 3.2.1.21) originating from almonds. The content of podophyllotoxin in undifferentiated, dark-grown cell suspension cultures of *C. drummondii*, generally reached levels of only 0.02% on a dry weight basis, during 2 years of subculturing. In the cell suspensions of *C. drummondii*, 80-90% of the podophyllotoxin was present in its β-D-glucosidic form. Generally, the cleavage of glucosides by hydrolysis at higher temperatures can not be excluded, and consequently artificially formed podophyllotoxin will be measured. For this reason, we determined the influence of the standard drying method (at 60 °C) on the podophyllotoxin-β-D-glucoside content with that of lyophilization, where no decomposition occurs. It appeared that in cell material 1-5% of this glucoside was converted into the aglucone after 24 h of drying at 60 °C, while for the needles 20-25%

was found. After correction for these losses, the glucoside content in the needles was around 32%, while Kier et al. (1963) reported that podophyllotoxin was only present as a glucoside. For determination of the podophyllotoxin-β-D-glucoside in needles, drying by means of lyophilization gave the most optimal results. The standard drying method did not significantly influence the aglucone/β-D-glucoside composition in cultured cells. In the spent culture medium no podophyllotoxin or its glucoside could be detected.

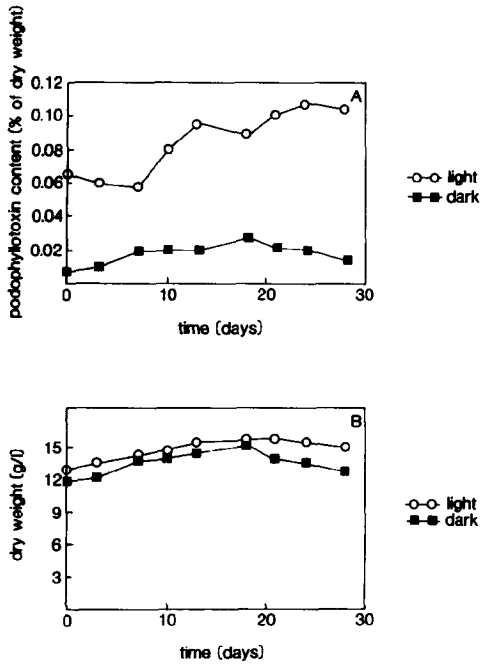

Fig. 2. Comparison of the podophyllotoxin (aglucone and glucoside) content (**A**) and dry weight changes (**B**) in cell suspension cultures derived from *Callitris drummondii*, grown under light and dark conditions.

The production of podophyllotoxin-β-D-glucoside in the suspensions was stimulated upon illumination. The podophyllotoxin levels in these suspensions were 4-6 times higher as compared with dark-grown cultures (Fig. 2A). Still 80-90% of the podophyllotoxin was present in the β-D-glucosidic form. The production rates calculated for light- and dark-grown cultures were 0.4 and 0.2 mg podophyllotoxin l^{-1} day^{-1}, respectively, present as β-D-glucoside. In addition, transfer of the dark-grown cell cultures to light, resulted in the afore mentioned higher levels already after two growth cycles. After the transfer back to the dark, reversed observations were done.

The dry weight increase was comparable under both conditions (Fig. 2B). Growth, in terms of increase of dry weight, occurred slowly: 20-50% of dry weight increase was obtained within one growth cycle of three weeks. Probably, this is typical for cell cultures

derived from conifers. This is also illustrated by the results of Berlin et al. (1984), who reported on the very slow growth of a cell culture of *Thuja occidentalis*, belonging to the Cupressaceae as well, where only 18-29% of fresh weight increase was obtained within a period of 18 days.

In the present study, it was shown that the illumination of cell cultures of *C. drummondii* stimulated the podophyllotoxin synthesis, but this is not necessarily a general effect. Recently, we reported that dark-grown cultures of *Podophyllum hexandrum* produced 2-3 times higher amounts of podophyllotoxin than light-grown cultures, up to 0.1% on a dry weight basis. (Van Uden et al. 1989; Chapter 2). Apparently, the effect of light on the biosynthesis of lignans depends on the plant species, culture conditions or other complex factors.

To our knowledge, this is the first report on podophyllotoxin-β-D-glucoside accumulation by undifferentiated cell cultures of *C. drummondii*. Cultures of *P. hexandrum* produced 0.1% podophyllotoxin (Van Uden et al. 1989; Chapter 2) and cultures of *Linum flavum* produced 0.73% 5-methoxypodophyllotoxin on a dry weight basis (Van Uden et al. 1990; Chapter 3). The cultures of *C. drummondii* grow slowly, consequently, the production rates are low. In this study, a rate of 0.4 mg l^{-1} day^{-1} was found for the light-grown cultures, while for dark-grown cultures of *P. hexandrum* a value of 1.3 mg l^{-1} day^{-1} (Chapter 2) and for light-grown cultures of *L. flavum* a value of 6.0 mg 5-MPT l^{-1} day^{-1} (Chapter 3) was calculated. Therefore, the cultures of *P. hexandrum* and *L. flavum* are considered to offer better prospects for the improvement of the production of podophyllotoxin and related compounds.

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CHAPTER 5

IMPROVEMENT OF THE PODOPHYLLOTOXIN PRODUCTION BY FEEDING OF PHENYLPROPANOIDS TO CELL CULTURES OF *PODOPHYLLUM HEXANDRUM* ROYLE.

(Published in Plant Cell Tissue and Organ Culture 23: 217-224 and Plant Cell Reports 9: 97-100)

ABSTRACT

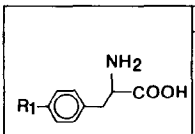
In order to improve the production of the cytotoxic lignan podophyllotoxin, seven phenylpropanoids from the phenylpropanoid biosynthesis routing and one related compound were fed to cell suspension cultures derived from the roots of *Podophyllum hexandrum* Royle. Only upon addition of coniferin, a stimulation of the podophyllotoxin accumulation was observed. Permeabilization using isopropanol, in combination with coniferin as a substrate, did not result in an additional increase in podophyllotoxin accumulation. Concentrations of isopropanol exceeding 0.5% (v/v) were found to be toxic for suspension-grown cells of *P. hexandrum*. When coniferin was fed in the presence of such isopropanol concentrations, β-glucosidase activity was still present, resulting in the formation of the aglucone coniferyl alcohol. In addition, podophyllotoxin was released into the medium under these permeabilization conditions.
Entrapment of *P. hexandrum* cells in calcium-alginate as such or in combination with the feeding of biosynthetic precursors, did not improve the podophyllotoxin production. Cell-free medium from suspension cultures at later growth stages incubated with coniferin, resulted in the synthesis of the lignan pinoresinol.
In parallel experiments, the use of β-cyclodextrin in feeding the poorly water-soluble precursor coniferyl alcohol to these cultures was studied. By complexation with β-cyclodextrin, a solution of 3 mM coniferyl alcohol could be fed, resulting in a 6-fold enhanced podophyllotoxin accumulation. The same concentration of non-complexed, suspended coniferyl alcohol had a lesser effect on the podophyllotoxin accumulation. β-Cyclodextrin itself was proven to be non-toxic for the cells. It did not influence the podophyllotoxin content and it was not metabolized or used as a carbon source by the cells. For comparison, coniferin, the water-soluble β-D-glucoside of coniferyl alcohol, was also fed in the same concentration. The effect of coniferin on the podophyllotoxin accumulation was ca. 5-fold stronger than that of coniferyl alcohol complexed with β-cyclodextrin.

INTRODUCTION

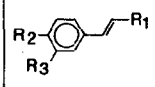
Etoposide (VP-16-213) and teniposide (VM-26) are two clinically applied semi-synthetic cytostatics, chemically prepared from podophyllotoxin (Holthuis 1988; Van Maanen et al. 1988). Podophyllotoxin belongs to the chemical group of lignans, which are dimerization products of phenylpropane (C6-C3) units, coupled by way of the β-carbons of their side chains (MacRae and Towers 1984; Pelter 1986). Podophyllotoxin is isolated from the rhizomes of *Podophyllum peltatum* or *Podophyllum hexandrum* (Berberidaceae)

(Jackson and Dewick 1984c). The availability of the plants is limited, principally because of a long juvenile phase and poor reproduction capacities (Rust and Roth 1981; Chuang and Chang 1987). Therefore, the biotechnological production of podophyllotoxin by means of plant cell tissue cultures, may be an attractive alternative.

Recently, we successfully initiated cell suspension cultures of *P. hexandrum*. Podophyllotoxin was accumulated up to 0.1% on a dry weight basis (Van Uden et al. 1989; Chapter 2). To our knowledge, precursor feeding studies using cell suspension with the aim to improve podophyllotoxin accumulation have not been performed so far. In the present study, we explored the potential of these cultures in feeding experiments. The phenylpropanoids involved were intermediates from the phenylpropanoid routing: L-phenylalanine, L-tyrosine, *trans*-cinnamic acid, *trans*-caffeic acid, *trans*-coumaric acid, *trans*-ferulic acid and coniferin. One structurally related compound, *trans*-3,4-methylenedioxycinnamic acid, was also tested. The structural formulas of these compounds are depicted in Fig. 1.



R_1		
H		L-phenylalanine
OH		L-tyrosine



R_1	R_2	R_3	
COOH	H	H	trans-cinnamic acid
COOH	OH	H	trans-coumaric acid
COOH	OH	OCH_3	trans-ferulic acid
COOH	OH	OH	trans-caffeic acid
COOH	$-OCH_2O-$		trans-3,4-methylenedioxy-cinnamic acid
CH_2OH	OGluc	OCH_3	coniferin

Fig. 1. Structural formulas of the precursors of podophyllotoxin used in this study.

The use of several organic solvents in order to accomplish permeabilization of plant cells has been reported (Rhodes 1985). Coniferin-feeding was combined with isopropanol with the aim to increase the formation of podophyllotoxin by freely suspended cells.

In addition, the cell suspension was immobilized in order to stimulate the production of podophyllotoxin after substrate feeding. Several successful attempts have been reported on the production of valuable compounds by immobilized plant cells (Brodelius and

Mosbach 1982; Wichers et al. 1983; Hall et al. 1988; Pras et al. 1988). The cells of *P. hexandrum* were entrapped in calcium alginate and a number of precursors were tested. Since freely suspended cells can release enzymes into the medium, dissolved substrates may be bioconverted extracellularly. Cell-free media were therefore checked on possible enzyme activities.

Several precursors are poorly soluble in aqueous media and consequently poorly water-soluble precursors have often been applied in two-phase systems. However, many of the plant cells used hardly converted precursors in the presence of organic phases, often due to a dramatic decrease of cell vitality (Beiderbeck and Knoop 1988). Another approach to solve this problem is to combine the advantages of apolar systems (higher solubility) and aqueous systems (compatibility with plant cells with respect to vitality) by carrying out bioconversions in the presence of clathrating agents, such as cyclodextrins (Woerdenbag et al. 1990).

In a parallel study therefore, coniferyl alcohol (Fig. 1), a key-precursor of the biosynthetic pathway of podophyllotoxin (Jackson and Dewick 1984b), has been chosen as a model substrate because of its very poor water-solubility.

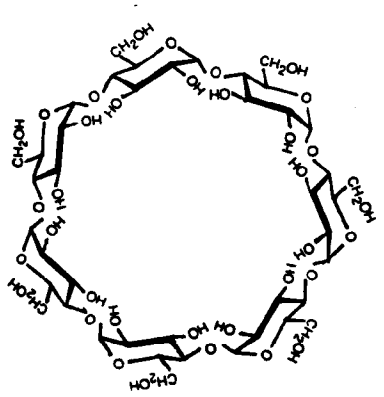


Fig. 2. Structural formula of β-cyclodextrin.

We investigated podophyllotoxin contents in *P. hexandrum* cell cultures, after feeding coniferyl alcohol, complexed with β-cyclodextrin (Fig. 2). For comparison, we fed the cultures with non-complexed, suspended coniferyl alcohol as well as with coniferin, the water-soluble β-D-glucoside of coniferyl alcohol. In addition, the influence of β-cyclodextrin on cell viability was monitored.

MATERIALS AND METHODS

CULTURE CONDITIONS AND CHARACTERISTICS

Cell suspensions of *Podophyllum hexandrum* Royle (Berberidaceae) derived from the roots, were grown as described previously (Van Uden et al. 1989; Chapter 2), here subculturing was done after a 3-week growth period. In the present study, the dark-grown cultures were used. The packed cell volume (PCV), pH, conductance and dry weight (DW) were routinely determined.

ENTRAPMENT

Freely suspended cells were immobilized in calcium alginate according to the procedure of Wichers et al. (1983), resulting in beads with a cell loading of 33% (w/v) and with an average volume of 84.9 µl.

PRECURSOR FEEDING

Bioconversions were carried out aseptically in Erlenmeyer flasks on a rotary shaker at 150 rpm at 26 °C. The precursors L-phenylalanine, L-tyrosine, *trans*-cinnamic acid, *trans*-caffeic acid, *trans*-ferulic acid, *trans*-coumaric acid and *trans*-3,4-methylene-dioxycinnamic acid were all purchased from Janssen Chimica. Coniferin, which is not commercially available, was a generous gift from Prof. Dr. K. Weinges, Heidelberg and Dr. J. Berlin, Braunschweig, both Germany.
All these compounds were added to the media in a standard concentration of 2.5 mM, prior to autoclaving. Cells in the stationary phase were washed once with medium and then inoculated at a packed cell volume of ca. 16%, resulting in final substrate concentrations of 2.1 mM. Media including 0.6, 1.0, 2.5, 4.8 and 9.5 mM coniferin as a substrate were tested. Immobilization experiments were carried out in Erlenmeyer flasks with 20 g beads in 100 ml medium.
In feeding experiments using calcium alginate-entrapped cells, the precursors L-phenylalanine, L-tyrosine, caffeic acid, ferulic acid and coniferin were tested.
Samples of ca. 10 ml were taken from the suspension-grown or immobilized cultures for a 3-4 week period.
The amount of substrate converted was calculated from the maximal difference in podophyllotoxin content between the control and the substrate-fed cultures. The bioconversion percentage was calculated as follows: substrate converted (mM)/ initial substrate concentration (mM) x 100%.
In order to determine conversion of coniferin in the medium, 2.0 ml of cell-free medium were combined with 2.0 ml of an aqueous 2.5 mM coniferin solution. In the control assay, fresh medium was used in stead of cell-free medium.

PRECURSOR FEEDING USING β-CYCLODEXTRIN

β-Cyclodextrin (AVEBE, Veendam, The Netherlands) is a cyclic oligosaccharide consisting of 7 glucose units, linked by α(1→4) glycosidic bonds (Fig. 2). Complex formation of coniferyl alcohol (Fluka, no. 27740) with β-cyclodextrin was achieved by autoclaving (20 min, 120 °C) these compounds in equimolar amounts, simultaneously with the cell culture media, resulting in complete dissolution of the coniferyl alcohol.
The increase in water-solubility of coniferyl alcohol, as a complex with β-cyclodextrin, was determined as described earlier (Higuchi and Connors 1965; Woerdenbag et al. 1990). The complex stability constant, K_C, was determined according to the method described by Selvidge and Eftink (1986). Sodium 4-(4-hydroxy-1-naphthylazo)-1-naphthalene sulfonate (a generous gift from Dr. Matsui, Shimane University, Japan) was used as the chromophoric ligand.

Each experiment was initiated by incubating 10 ml of packed cells of a 3-week-old cell suspension culture in 100 ml fresh medium. Coniferyl alcohol, as a β-cyclodextrin complex, was added in a concentration of 3 mM at the beginning of the growth cycle (day 0). For comparison, 3 mM coniferyl alcohol without β-cyclodextrin as well as 3 mM coniferin (coniferyl alcohol-β-D-glucoside) were added in analogous experiments. Control experiments were performed with standard-grown cell suspensions, either without or with 3 mM β-cyclodextrin.
During the three-week experiments, 10 ml samples of cell suspension were taken at regular intervals.
β-Cyclodextrin concentrations in the culture medium and in the aqueous extract of the dried and powdered cell material were determined according to Vikmon (1982). Briefly, samples were 30-fold diluted with water. To 1.0 ml sample, 1.0 ml alkaline phenolphthalein solution (phenolphthalein 5 x 10^{-5} M, sodium carbonate 0.01 M in water) was added and the sample was mixed. The UV-absorption at 550 nm was measured. The difference in absorption as compared with reference sample (water) was determined. The β-cyclodextrin concentration was calculated from a calibration curve prepared from samples with known β-cyclodextrin concentrations.

PERMEABILIZATION

Permeabilization of freely suspended cells was carried out using the organic solvent isopropanol (2-propanol, Merck). The isopropanol was added aseptically via a 0.2 μm filter (Millipore) after autoclaving the media. Concentrations of 0.5, 1.0, 2.5, 5.0 and 10% (v/v) were used.

RESPIRATION ACTIVITY

The respiration activity of calcium alginate-entrapped cells was determined by means of measuring the oxygen consumption, in reference to freely suspended cells. Oxygen was measured with an oxygen electrode (O_2-meter CG 867, Schott). The consumption of 2.0 g beads or 1.0 g freely suspended cells in oxygen-saturated growth medium in a 23.0 ml vessel was monitored under stirring at 20 °C during 30 min. The oxygen consumption is expressed as mg O_2 consumed per h per g fresh weight cells (mg O_2 $h^{-1}g^{-1}$FW).

β-GLUCOSIDASE ACTIVITY (E.C. 3.2.1.21)

Portions of ca. 0.3 g, fresh weight, of *P. hexandrum* cells were ground with quartz in liquid nitrogen and transferred to 2.0 ml 0.5 M sodium phosphate buffer, pH 5.0, containing ca. 100 mg Dowex (Sigma No. 1x2-100). The extract was centrifuged at 17,000 g for 10 min at 4 °C. The supernatant, designated as the soluble protein fraction, was directly used for activity measurement, while the pellet (cell fractions) was resuspended in 2.0 ml 0.5 M phosphate buffer, pH 5.0. One ml of the supernatant or resuspended pellet was added to 4.0 ml of an aqueous 2.5 mM solution of coniferin. To measure β-glucosidase activity in cell-free medium, a cell suspension sample was centrifuged at 17,000 g for 10 min at 4 °C. One ml of supernatant plus 1.0 ml 0.5 M phosphate buffer, pH 5.0, were added to 4.0 ml 2.5 mM aqueous coniferin solution. Standard incubations were done for 1 h at 40 °C. For determination of the coniferyl alcohol content in the enzyme fractions, samples were taken at t=0 min. The reaction was stopped by adding 2.0 ml of the assay mixture to 4.0 ml dichloromethane, followed by immediate vortexing in order to extract the liberated coniferyl alcohol. Two ml of the dichloromethane phase were evaporated to dryness and the remaining residue was redissolved in 1.0 ml methanol. These extracts were analyzed by means of HPLC-UV (see below). The β-glucosidase activity is expressed as mmol coniferyl alcohol formed per s per kg protein (mkat kg^{-1} protein) in medium and supernatant or mmol coniferyl alcohol formed per h per kg fresh weight (mmol h^{-1} kg^{-1} FW) in cell fractions.

PEROXIDASE ACTIVITY (E.C. 1.11.1.7)

Peroxidase activity was measured in the soluble protein and pellet fraction of *P. hexandrum* cells, as well as in cell-free medium. These samples were obtained according to the procedure described under the section β-GLUCOSIDASE ACTIVITY. Here, a 0.1 M phosphate buffer, pH 7.0, was used. The activity was determined spectrophotometrically according to the method of Maehly and Chance (1954).
Peroxidase activity is expressed as mmol guaiacol consumed per s per kg protein (mkat kg^{-1} protein) in medium or supernatant, or mmol guaiacol consumed per h per kg fresh weight (mmol h^{-1} kg^{-1} FW) in cell fractions.

PROTEIN CONTENT

The protein content was determined according to the method of Bradford (1976) using bovine serum albumin (BSA, Sigma no. A7638) as the standard protein.

EXTRACTION PROCEDURE

Crude extracts to be used for the several methods of analysis were prepared to a described procedure (Van Uden et al. 1989; Chapter 2), immobilized cells were dried by means of lyophilization.

ANALYSIS BY MEANS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Podophyllotoxin was analyzed and detected using the HPLC-UV system as reported previously (Van Uden et al. 1989; Chapter 2). Production rates were calculated as described in Chapter 2. Coniferyl alcohol was measured in the same extracts that were used to determine podophyllotoxin, at 290 nm using a Lichrosorb RP-18 column at a flow rate of 0.3 ml min^{-1}. A reference solution of 0.1 mg ml^{-1} coniferyl alcohol (Fluka, no. 27740) in methanol was used.
Analysis of coniferin was performed using a Chrompack Nucleosil 5C-18 column (100 x 3 mm i.d.). The water phase of the extraction and the spent medium were directly used for the analyis of coniferin. The mobile phase consisted of methanol/water (3:7), the flow rate was 0.15 ml min^{-1} and the detection was at 230 nm. A coniferin solution of 0.01 mg ml^{-1} in water was used as the standard.

MASS SPECTROMETRY

Mass spectrometric analysis (direct sampling) of cell-free medium extracts were performed under the conditions as described (Van Uden et al. 1989; Chapter 2) and revealed the presence of pinoresinol. The following values were found: m/z, relative intensities (%): [M^+] 358 (7), 205 (9), 163 (26), 152 (35), 151 (100), 137 (77).
In addition, a chemical ionization using OH^- was carried out. The samples were introduced into the mass spectrometer by means of direct sampling and identified at 100 eV. For pinoresinol an $[M-H]^-$ value of 357 was found.

RESULTS AND DISCUSSION

PRECURSOR FEEDING TO FREELY SUSPENDED CELLS

Seven intermediates from the phenylpropanoid pathway and one related compound were tested as substrates with freely suspended cells of *Podophyllum hexandrum* Royle. It appeared that 2.1 mM concentrations of cinnamic acid, coumaric acid and 3,4-methylenedioxycinnamic acid were too toxic for the cells, as determined on basis of packed cell volume, dry weight, pH and conductance. None of these precursors resulted in enhanced podophyllotoxin levels. The intermediates L-phenylalanine, L-tyrosine, ferulic acid, caffeic acid and coniferin did not result in any effect on these culture characteristics. Phenylalanine, tyrosine, ferulic acid and caffeic acid administration resulted even in a decrease of the podophyllotoxin content.

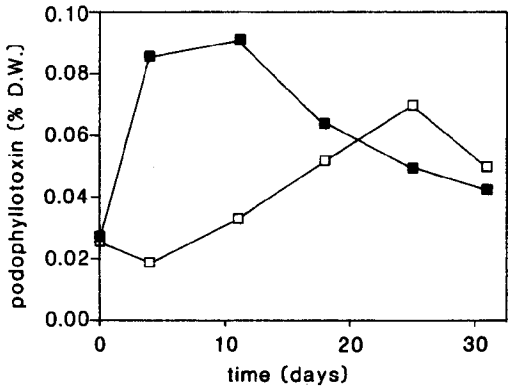


Fig. 3. Time course of the podophyllotoxin content on a dry weight basis of a suspension culture of *P. hexandrum*; standard-grown (□) and after feeding of 2.1 mM coniferin (■).

From the precursors tested, only coniferin was found to exhibit a positive and reproducible effect on the podophyllotoxin accumulation. The highest calculated bioconversion percentage was 1.88% at a 2.1 mM coniferin concentration. In Fig. 3 it is shown that in coniferin-fed cultures of *P. hexandrum* a 4.5 times higher podophyllotoxin content was found at day 4, compared to untreated cultures, respectively 0.085% and 0.018% on a dry weight basis. A high increase factor does not imply a high absolute podophyllotoxin content. For the coniferin-fed culture a production rate of 1.2 (11 days) could be calculated, while for the standard culture a value of 0.5 mg podophyllotoxin l^{-1} day^{-1} (25 days) was found. The podophyllotoxin contents (production rates) found for standard-grown cells in this study were lower than those published earlier (Van Uden et al. 1989; Chapter 2). Apparently this cell line is subjected to fluctuations in accumulation levels. Remarkable was that the highest contents in standard-grown cultures were at the stationary phase of the growth cycle, while coniferin-fed cultures on the contrary, reached

maximal values already in the early lineair phase. The addition of coniferin mainly resulted in gain of time, which is also reflected in the production rates, the final podophyllotoxin contents were not markedly increased. Coniferin was not detectable in standard-grown cells and together with this rapid production of podophyllotoxin, this might point to an intracellular substrate limitation.

As is shown in Fig. 4, the optimal substrate concentrations lie in the range of 0.8 to 2.1 mM coniferin. Only at concentrations exceeding 4.0 mM, growth inhibitory effects occurred, indicating that coniferin is a rather non-toxic substrate.

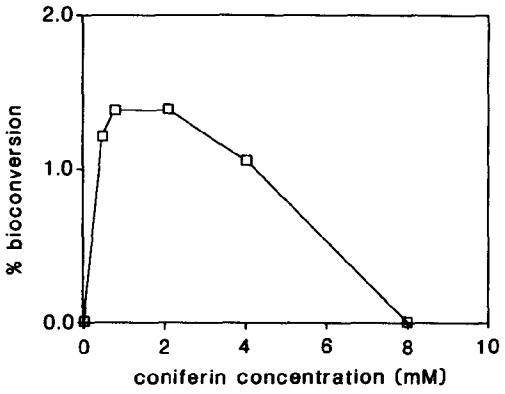


Fig. 4. Relationship between the coniferin concentration and its bioconversion percentage, after administration to suspension-grown cultures of *P. hexandrum*.

The coniferin administered to the cell suspensions disappeared rapidly from the medium, 55% of the substrate vanished from the medium within 2 h. After 24 h only 1% was still present extracellularly. However, maximally only 1.8% of originally administered coniferin was endogenously found at that moment. Since the aglucone of this substrate, coniferyl alcohol, could not be detected intra- or extracellularly, it can be suggested that 98% was converted into unknown products. From the three alcohols, known to be incorporated into lignin, the constituent of cell walls of various types of plants, coniferyl alcohol is most efficiently used (Freudenberg and Harkin 1963; Hahlbrock 1977; Luckner 1986). Lignin might be a possible 'catcher' of supplied coniferin (via coniferyl alcohol) in bioconversion experiments, as has also been proposed by Stöckigt and Klishies (1977).

PERMEABILIZATION OF FREELY SUSPENDED CELLS

In order to improve the availability of coniferin for bioconversion by freely suspended cells, this substrate was supplied in combination with isopropanol as a permeabilizing agent. All isopropanol concentrations exceeding 0.5% (v/v) inhibited growth and caused an increase in conductivity of the cell-free medium, suggesting cell lysis, as is shown in

Fig. 5. It has been reported, that even mild treatments with the strongly related organic solvent n-propanol, resulted in severe growth inhibition (Berlin et al. 1989). The combination of coniferin-feeding and cell-permeabilization did not lead to an additional increase of podophyllotoxin accumulation. At very high isopropanol concentrations, 5 or 10%, coniferyl alcohol was found in the medium, indicating the hydrolysis of coniferin to its aglucone (Fig. 6). When 10% isopropanol was used, all coniferin was converted into coniferyl alcohol after 48 h, 5% isopropanol resulted in a lower conversion percentage of coniferin.

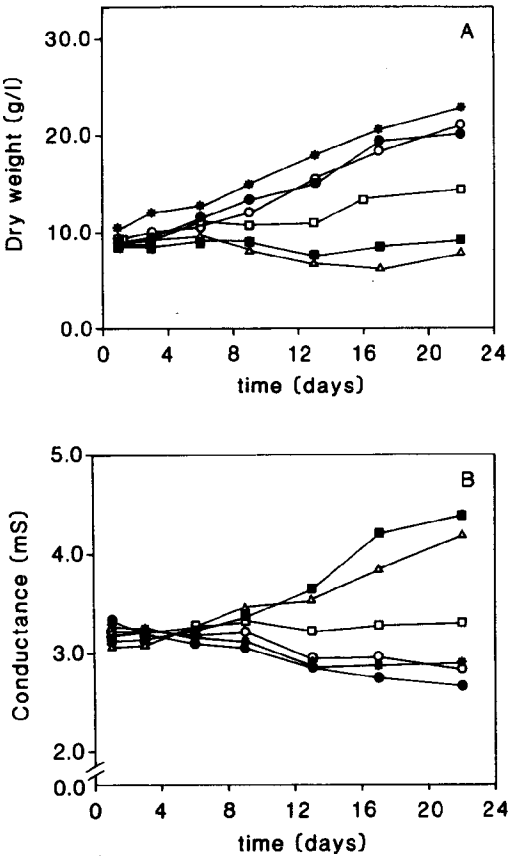


Fig. 5. Dry weight (**A**) and conductance (**B**) changes under various permeabilization conditions. Control (●); 2.5% (v/v) isopropanol (■); 2.1 mM coniferin (✱); 2.1 mM coniferin + 0.5% (v/v) isopropanol (○); 2.1 mM coniferin + 1.0% (v/v) isopropanol (□); 2.1 mM coniferin + 2.5% (v/v) isopropanol (△).

On the contrary, negligible amounts of coniferyl alcohol were detected in the medium of a coniferin-fed and isopropanol-untreated culture.

It seems likely that this hydrolysis of coniferin into the aglucone takes place at the cell wall/plasmalemma: β-glucosidase activity was measured in the cell fraction and not in the

medium, while coniferyl alcohol was detected in the medium and only for trace amounts intracellularly. Isopropanol did not influence the total β-glucosidase activity during the experiment (9-12 mmol h^{-1} kg^{-1} FW), but remarkably was the increase of activity associated with the soluble protein fraction, from ca. 1 to 2.5 mkat kg^{-1} protein and from ca. 1 to 4.5 mkat kg^{-1} protein, for 5% and 10% isopropanol respectively. These results indicate that the hydrolysis of coniferin was due to the increased activity in the soluble fraction under these permeabilization conditions.

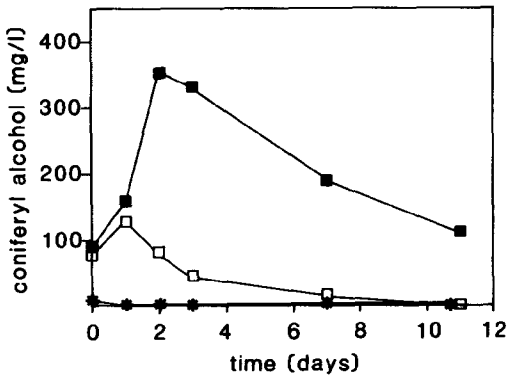


Fig. 6. Coniferyl alcohol in the cell-free medium after feeding 2.1 mM coniferin to suspension cultures of *P. hexandrum*: no permeabilization (✱), permeabilization with 5% (v/v) isopropanol (□), and 10% (v/v) isopropanol (■).

Although cell growth ceased, a β-glucosidase was still able to convert coniferin into its aglucone. Apparently, severely stressed plant cells are able to convert administered intermediates by way of active enzyme systems.

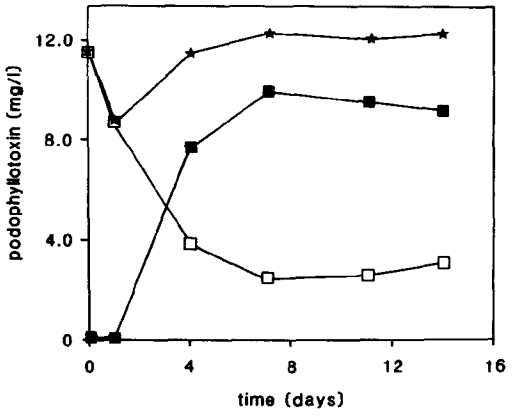


Fig. 7. Time course of extracellular (■), intracellular (□) and total (✱) podophyllotoxin content of a suspension culture of *P. hexandrum* after feeding 2.1 mM coniferin in combination with 5% (v/v) isopropanol.

As an additional effect, the endogeneously present podophyllotoxin was released into the medium after isopropanol treatment. For example, when 5% isopropanol was used, 50% of podophyllotoxin was released after 3 days, the release being complete after 7 days (Fig.

7). This release might be a helpful tool for the isolation of intracellularly stored podophyllotoxin, although the method is cell-destructive.

PRECURSOR FEEDING TO ENTRAPPED CELLS

In some studies it has been observed that precursor feeding to immobilized cells resulted in an increased production of secondary products when compared to freely suspended cells under the same conditions (Brodelius and Mosbach 1982; Wichers et al. 1983; Rhodes 1985; Hall et al. 1988; Pras et al. 1988). In a recent study, calcium alginate proved to be a suitable matrix for cell entrapment in combination with precursor feeding (Pras et al. 1989). Therefore, the bioconversion experiments were carried out using calcium alginate-entrapped cells of *P. hexandrum*. Only precursors that were non-toxic for freely suspended cells were tested: L-phenylalanine, L-tyrosine, caffeic acid, ferulic acid and coniferin. The immobilization procedure itself did not lead to an improved podophyllotoxin accumulation, the synthesis of podophyllotoxin even ceased. Metabolic behaviour of cells may be altered after entrapment. Analogously, for cells of *Mucuna pruriens* it has been reported that the endogenous L-DOPA production stopped completely upon immobilization (Wichers et al. 1983).
The oxygen consumption of freely suspended cells of *P. hexandrum* was 0.30 mg O_2 h^{-1} g^{-1} FW. The respiration activity decreased immediately after the entrapment to a value of 0.16 mg O_2 h^{-1} g^{-1} FW, which remained stable during the experiments. Possibly, the entrapped state is a stress environment for the cells, in which they are not able to synthesize podophyllotoxin anymore. Feeding of L-phenylalanine, L-tyrosine, ferulic acid and caffeic acid did not result in an enhanced production of podophyllotoxin. Also with coniferin, in contrast with freely suspended cells, no podophyllotoxin increase was measured. This substrate disappeared completely from the medium within 7 days, which is much slower than measured for freely suspended cells.

EXTRACELLULAR ENZYME ACTIVITIES

Since the release of enzymes from plant cells into the medium can not be excluded (Sticher et al. 1981; Rhodes 1985), coniferin was added to cell-free medium in order to study its possible extracellular bioconversion. Mass spectrometric analysis of cell-free medium extracts revealed the presence of the lignan pinoresinol (Fig. 8), which showed the same fragmentation pattern as was found by Duffield (1967). This extracellularly formed product was only present in later growth stages. Theoretically, the synthesis of pinoresinol in cell-free medium can only be achieved when a β-glucosidase activity and a peroxidase activity are present. Glucosidase activity is required, since glucosides are not substrates for peroxidase (Hösel and Todenhagen 1980). Peroxidase activity is essential

for the formation of coniferyl alcohol radicals, needed for further biosynthesis into lignans (Stöckigt and Klishies 1977). Activities of both enzymes could indeed be measured in the cell-free medium at the stationary phase, and fluctuated around 4.2 mkat kg^{-1} protein for peroxidase and around 0.6 mkat kg^{-1} protein for β-glucosidase activity.

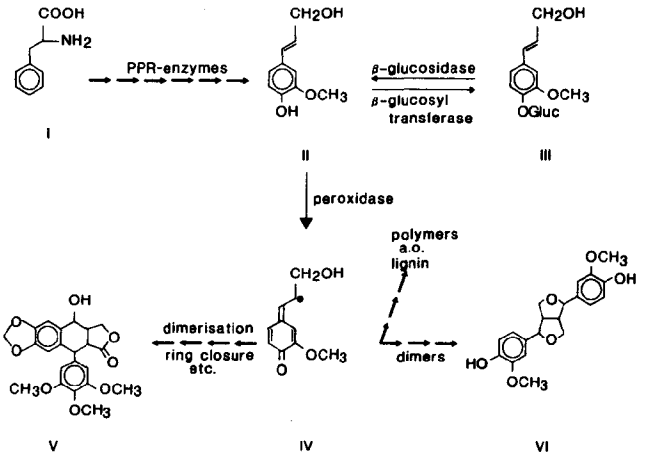


Fig. 8. A simplified scheme of the biosynthesis of podophyllotoxin. From phenylalanine (I), coniferyl alcohol (II) is synthesized by the action of phenylpropanoid (PPR) enzymes. This can be converted into coniferin (III) and vice-versa. A peroxidase converts (II) to coniferyl alcohol radicals (IV), which are coupled and synthesized further to lignans such as podophyllotoxin (V) or pinoresinol (VI).

In presence of these enzymes, the formation of pinoresinol from coniferyl alcohol, via coniferin, has been reported several times (Freudenberg and Rasenack 1953; Erdtman 1955; Freudenberg and Harkin 1963; Harborne 1980; Luckner 1986). Thus, in order to avoid unwanted extracellular bioconversion of coniferin, this substrate has to be added to *P. hexandrum* cells in the early growth stage.

PRECURSOR FEEDING USING β-CYCLODEXTRIN

Cyclodextrins are cyclic oligosaccharides that are able to form inclusion complexes with a variety of apolar ligands. Through complexation the physical-chemical properties of the ligands are changed, including their solubility in aqueous solution (Szejtli 1982). At 26 °C the water-solubility of coniferyl alcohol increased from 0.15 mM without, to a maximum of about 3.4 mM with β-cyclodextrin, in a molar ratio of 1:1. A stable complex was formed, with a K_c of 1360 M^{-1}. Cells of *P. hexandrum*, endogenously accumulated podophyllotoxin in concentrations ranging from 0.001 to 0.002% (production rate 0.02 mg l^{-1} day^{-1}, 13 days), calculated on a dry weight, during their growth cycle (Fig. 9). The podophyllotoxin concentrations, as measured in the control experiments, are somewhat

lower than those published earlier from the same undifferentiated cell line (Van Uden et al. 1989; Chapter 2). Apparently, the cell line is subject to fluctuations and not stable for a longer period in this respect, which is also demonstrated in terms of production rates. During maintenance of these cultures, the rates decreased from 1.3, via 0.5 to 0.02 mg podophyllotoxin l^{-1} day^{-1}.

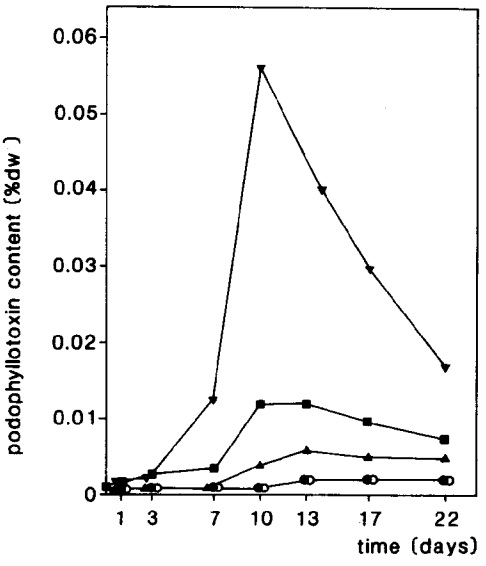


Fig. 9. Time course of the podophyllotoxin content, expressed as percentage of dry weight, in suspension-grown cultures of *P. hexandrum*. Control (●); 3 mM β-cyclodextrin (○); 3 mM coniferyl alcohol/β-cyclodextrin complex (■); 3 mM coniferyl alcohol (▲); 3 mM coniferin (▼).

The precursor-feeding experiments were performed with the highest possible coniferyl alcohol/β-cyclodextrin concentration. Feeding of 3 mM coniferyl alcohol, dissolved in the culture medium as a β-cyclodextrin complex, resulted in enhanced podophyllotoxin accumulation, with a maximum of 0.012% on day 10 of the growth cycle (production rate 0.2 mg podophyllotoxin l^{-1} day^{-1}, 10 days). Non-complexed coniferyl alcohol, suspended in the medium in a concentration of 3 mM, also enhanced the podophyllotoxin production, but only to a maximum of 0.006% (production rate 0.1 mg l^{-1} day^{-1}, 13 days). Feeding 3 mM coniferin caused the largest increase, with a maximum of 0.056% on day 10 (Fig. 9), and a corresponding production rate of 0.8 mg podophyllotoxin l^{-1} day^{-1}. This was in agreement with the former results, found in parallel studies, in which only water-soluble precursors were tested.

The accumulation pattern of podophyllotoxin, after adding only β-cyclodextrin to the culture medium, did not differ from the control conditions. The culture characteristics, *i.e.* cell growth in terms of dry weight, pH and conductivity, were not altered by any of the additions. The β-cyclodextrin concentration in the culture medium remained unchanged during the whole growth cycle and no β-cyclodextrin could be detected intracellularly.

This indicates that the oligosaccharide was not metabolized by plant cell enzymes or used as a carbon source. Moreover, it did not penetrate the plant cells.

The lignan biosynthesis proceeds via a radical mediated dimerization reaction of two phenylpropane units, such as coniferyl alcohol (Pelter 1986). Coniferyl alcohol originates from coniferin, which looses glucose by specific β-glucosidase activity in the cultures (Freudenberg 1965). From the two precursors used, only coniferyl alcohol is commercially available.

It was found that the effect of coniferin was more pronounced than that of the coniferyl alcohol/β-cyclodextrin complex. Apparently, it is not advantageous in this case to feed a precursor, more closely related to the radical coupling reaction (coniferyl alcohol) than a substrate that first has to undergo cleavage of its sugar moiety (coniferin). In this respect, it has been postulated that intermediates of the lignan and lignin biosynthesis are carried in a hydrophilic, soluble form to the site of dimerization and polymerization, respectively (Hahlbrock 1977).

No podophyllotoxin could be detected in the culture medium, indicating that it was only present intracellularly and not excreted into the medium. Under control conditions, cells contained only 2 μg coniferyl alcohol per g dry weight. After feeding the β-cyclodextrin/substrate complex, an increased coniferyl alcohol content was found, 35 and 13 μg g^{-1}, respectively on day 1 and 3 of the growth cycle. In later stages of the growth cycle the low control levels were measured again. From the culture medium coniferyl alcohol had vanished within 1 day, despite this rapid disappearance, no direct podophyllotoxin formation was measured.

The direct benefit, with respect to a higher podophyllotoxin production, was probably obtained from the fact that coniferyl alcohol is delivered to the cells in a dissolved state, due to complexation with β-cyclodextrin. This may lead to an increased uptake of the precursor in the cells, finally resulting in an increased bioconversion, as compared with the non-complexed precursor. This observation seems to be confirmed by the relatively efficient incorporation of the readily soluble analog coniferin. At present, a lack of knowledge exists on the underlaying mass transfer processes that take place near the cell envelope. Recently, we successfully applied β-cyclodextrin to solubilize the steroid hormone 17β-estradiol, that subsequently was *ortho*-hydroxylated efficiently by a phenoloxidase from *Mucuna pruriens* cell cultures (Woerdenbag et al. 1990). In contrast with this one step bioconversion, the lignan routing is much more complex and several enzymatic steps and interconversions are involved, probably resulting in lower product yields.

FINAL CONCLUSIONS

From the results presented in this study, it may be concluded that the use of coniferin as a precursor increases the podophyllotoxin production of freely suspended cells of *P. hexandrum*. This conclusion fits in the biosynthesis scheme of podophyllotoxin, which has been based upon previous reports (Erdtman 1955; Jackson and Dewick 1984b; Pelter 1986) (Fig. 8). On the other hand, since no labelling experiments were performed, an indirect effect of coniferin can not be excluded. Improvement of the podophyllotoxin production could not be achieved by permeabilization or entrapment.

Precursors other than coniferin did not result in increased podophyllotoxin contents in our undifferentiated cell cultures, but on the other hand it is known that they can be incorporated into podophyllotoxin using intact plants (Ayres 1969; Ayres et al. 1981; Jackson and Dewick 1984a).

In addition, feeding lipophilic precursors of the lignan biosynthesis as a β-cyclodextrin complex, offers a possibility to enhance the podophyllotoxin content in cell cultures of *P. hexandrum*. In order to solubilize organic compounds, cyclodextrins offer perspectives in plant cell biotechnology. By their application very smooth bioconversion conditions are created with respect to cell viability. They may be used to facilitate precursor-feeding of poorly water-soluble intermediates or not naturally occurring compounds. Moreover, they open up perspectives to feed labelled lipophilic compounds, to be incorporated in secondary metabolites, in order to study a biosynthetic pathway.

Despite the positive results on the production of podophyllotoxin by feeding of coniferin and its aglucone, the biotechnological production of this lignan forms no commercial alternative yet. Since the podophyllotoxin accumulation in *P. hexandrum* cell suspensions appeared to be unstable and low contents were measured after a longer period of maintenance, it was decided to emphasize the further studies on the production of 5-methoxypodophyllotoxin by cell cultures of *L. flavum*.

ACKNOWLEDGEMENTS

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CHAPTER 6

THE ROLE OF CONIFERIN IN THE PRODUCTION OF 5-METHOXYPODOPHYLLOTOXIN BY CELL SUSPENSION CULTURES OF *LINUM FLAVUM* L. AND ITS ISOLATION FROM A HIGH-PRODUCING LINE.

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ABSTRACT

Cell suspensions of *Linum flavum* L. contained large amounts (2 g l^{-1}) of the glucoside coniferin, which was accumulated endogenously up to 12.4% on a dry weight basis. Callus material contained 5.6%, while in leaves of *in vitro* grown plantlets, the origin of the callus and suspension cultures, no coniferin could be detected. Leaf, callus and suspension material was compared for metabolite accumulation and enzyme activities involved. High coniferin contents corresponded with low 5-methoxypodophyllotoxin (5-MPT) levels. A reciprocal relationship between β-glucosidase activity and coniferin accumulation was found. No relationship between peroxidase activity and the accumulation of coniferin or 5-MPT could be demonstrated. Finally, a rapid and efficient isolation procedure for coniferin is described.

INTRODUCTION

It has been reported previously that cell cultures of *Linum flavum* L. (Linaceae) can produce the coniferyl alcohol-β-D-glucoside coniferin, see Fig. 1 (Berlin et al. 1986, 1988). Coniferin owes its name to its appearance principally in conifers, other names include laricin and abietin (Karrer 1976).

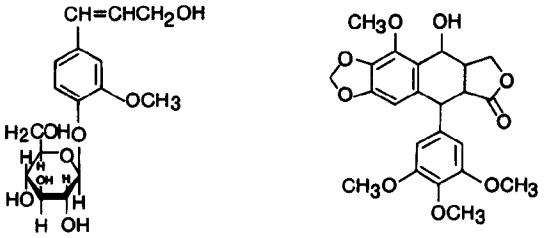

Fig. 1. Chemical structures of coniferin (**A**) and 5-methoxypodophyllotoxin (**B**).

Coniferin as well as the aglucone coniferyl alcohol occur in the well-known phenylpropanoid pathway (Jackson an Dewick 1984).

Apart from coniferin production, the accumulation of the lignan 5-methoxypodophyllotoxin (5-MPT) (Fig. 1) has been described (Berlin et al. 1986, 1988; Van Uden et al. 1990a, Chapter 3; Wichers et al. 1990). The more complex structure of 5-MPT, which is a dimerization product of phenylpropane units, also originates from the phenylpropanoid pathway.

Coniferyl alcohol, the aglucone corresponding to coniferin, is an important precursor of the cell-wall constituent lignin (Freudenberg 1965; Hahlbrock 1977). Recently, it was demonstrated that coniferin stimulates the accumulation of podophyllotoxin, either through direct incorporation, or through an indirect effect. This was demonstrated in feeding experiments with cell cultures of *Podophyllum hexandrum* (Van Uden et al. 1990b; Chapter 5; Woerdenbag et al. 1990).

Two important enzymes involved in metabolizing coniferin in the biosynthesis pathway are β-glucosidase and peroxidase (Freudenberg 1963, 1965; Harkin and Obst 1973; Hahlbrock 1977; Stöckigt and Klishies 1977).

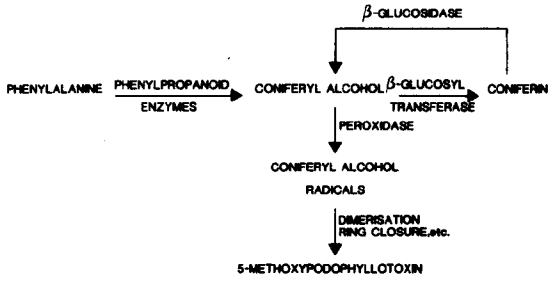

Fig. 2. Proposed and simplified scheme for the biosynthesis of 5-methoxypodophyllotoxin via coniferin.

The β-glucosidase converts coniferin into coniferyl alcohol, whereas peroxidase is involved in the synthesis of coniferyl alcohol radicals prior to dimerization (lignans) or polymerization (lignins) reactions. A proposed and simplified biosynthesis scheme is depicted in Fig. 2.

In the present study the relationship between coniferin, coniferyl alcohol and 5-MPT accumulation and the mentioned enzymes was investigated and an isolation procedure for coniferin was developed.

MATERIALS AND METHODS

PLANT MATERIAL, CULTURE CONDITIONS AND SAMPLING

Plantlets, callus and suspension cultures of *Linum flavum* L. (Linaceae) were obtained and grown as described recently (Van Uden et al. 1990a; Chapter 3). For the cell line used in this study, callus and suspensions were grown on an MS (Murashige and Skoog 1962) medium supplemented with 4% (w/v) sucrose and 1 mg l^{-1} of the phytohormones indole-3-acetic acid and 6-benzylaminopurine. Plantlets and calli were transferred to fresh medium every 4 weeks, while cell suspensions were subcultured by adding 100 ml of a 2-week-old cell culture to 200 ml of fresh medium. Cell cultures have been maintained under these conditions over a period of 2 years in our laboratory. For upscaling purposes with the aim to isolate coniferin, 2.1 l suspension was grown in a 3-l Erlenmeyer flask and were subcultured under the same conditions as the standard cultures. Samples of leaf and callus material were taken at the moment of subculturing, while cell suspension samples were taken during the growth cycle at regular intervals.

EXTRACTION PROCEDURE

The samples were dried by lyophilization, or at 60 °C for 24 h. It is known that glucosidic compounds are easily hydrolyzed at higher temperatures. The choice of the drying method was found to be crucial with respect to coniferin and coniferyl alcohol contents. When samples were dried for 24 h at 60 °C, coniferin was partially cleaved to its aglucone and glucose. Coniferin losses varied between 10-80%. Therefore we choose for lyophilization as the standard drying method.
Coniferyl alcohol and 5-MPT were extracted according to a previously described procedure (Van Uden et al. 1990a; Chapter 3). To determine coniferin contents in calli and suspension-grown cells, 0.1 ml of the water phase of the extraction was diluted 100 times with water, while the water layer of leaf extractions was diluted 10 times. All extracts were centrifuged for 2 min at 10,000 g (Eppendorf) prior to analysis by means of HPLC.

ANALYSIS BY MEANS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

5-MPT was analyzed and detected using the HPLC-UV system as reported previously (Van Uden et al. 1990a; Chapter 3). As a reference solution 0.1 mg ml^{-1} authentic 5-MPT in methanol was used. The 5-MPT was a generous gift from Dr. H.J. Wichers, TNO-ITC, Zeist, The Netherlands.
Coniferyl alcohol was measured at 290 nm using a Lichrosorb (Chrompack) RP-18 column (100 x 3 mm i.d.) equipped with a guard column (Chrompack) and methanol/water (4:6) as the eluent, flow rate: 0.3 ml min^{-1}, or at the conditions as described above for 5-MPT. A 0.1 mg ml^{-1} solution of coniferyl alcohol (Fluka) in methanol was used as the standard.
Analysis of coniferin was performed using a Chrompack Nucleosil 5C-18 column (100 x 3 mm i.d.), which was equipped with a guard column. The mobile phase consisted of methanol/water (3:7), the flow rate was 0.15 ml min^{-1} and the detection at 230 nm. A coniferin solution in water of 0.01 mg ml^{-1} was used as the standard. The production rate was calculated a described in Chapter 2. Coniferin was a generous gift from Prof. Dr. K. Weinges, Heidelberg, Germany and Dr. J. Berlin, Braunschweig, Germany.

β-GLUCOSIDASE ACTIVITY (E.C. 3.2.1.21)

Portions of 0.2-0.3 g fresh weight of leaf, callus or suspension-grown cells were ground with quartz in liquid nitrogen followed by a procedure as described by Van Uden et al. (1990b; Chapter 5). The β-glucosidase activity is expressed as mmol coniferyl alcohol formed per s per kg protein (mkat kg^{-1} protein) in medium or supernatant, or mmol coniferyl alcohol formed per h per kg fresh weight (mmol h^{-1} kg^{-1} FW) in cell fractions.

PEROXIDASE ACTIVITY (E.C. 1.11.1.7)

Peroxidase activity was measured in the soluble protein and pellet fraction of *L. flavum* leaf, callus and suspension samples. These fractions were prepared according to a previous procedure (Van Uden et al. 1990b; Chapter 5). Peroxidase activity is expressed as mmol guaiacol consumed per s per kg protein (mkat kg^{-1} protein) in medium or supernatant, or mmol coniferyl alcohol formed per h per kg fresh weight (mmol h^{-1} kg^{-1} FW) in cell fractions.

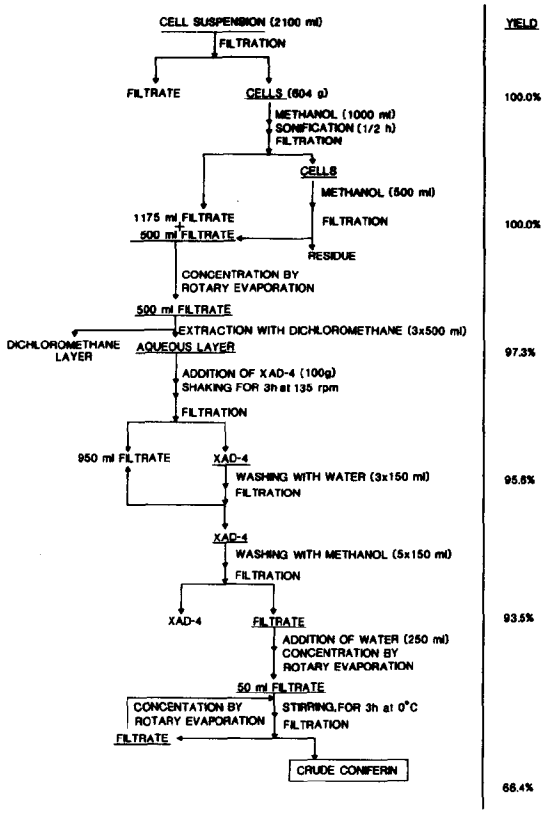

Fig. 3. Scheme for the isolation of coniferin from suspension cells of *L. flavum*. In the right column coniferin yields are indicated as a percentage of the initial amount.

PROTEIN CONTENT

The protein content was determined according to the method of Bradford (1976) using bovine serum albumin (Sigma no. A 7638) as the standard protein.

ISOLATION OF CONIFERIN AND CONFIRMATION OF ITS IDENTITY

In order to isolate coniferin, fresh or lyophilyzed cells of suspension cultures grown in 3-l Erlenmeyer flasks, were used.
The isolation procedure of coniferin from fresh cells was performed according to the scheme in Fig. 3. Lyophilized cells were sonificated for 1 h in 2 l methanol (80%; v/v) per 100 g, followed by the same extraction as described for fresh cells.
The Serdolit® XAD-4 adsorbent resin (300-1000 μm) was purchased from Serva (no. 42831), and regenerated by shaking during 24 h with methanol and water, respectively.
The pale-yellow, crude coniferin was recrystallized by redissolving coniferin in hot water to a concentration of 1 g per 20 ml, followed by filtration and subsequent stirring for 3 h at 0 °C. The white, needle-like crystals were collected by suction filtration and dried under vacuum at room temperature.
The isolated as well as authentic coniferin were analyzed using a gas chromatograph coupled with a mass spectrometer (Finnigan, 4500 GC-MS). Introduction into the GC-MS was done by means of solid probe. A fused silica column (25 m x 0.25 mm i.d.) was used, with helium as the carrier gas. The identification was by means of electron impact at an ionisation energy of 70 eV. In addition, ultraviolet-spectra were recorded on a Shimadzu Spectronic (210-UV) spectrophotometer and infrared-spectra (KBr-discs) on a Perkin-Elmer (577) infrared spectrophotometer.
Coniferin; m/z, relative intensities (%): [M^+] 180 (100), 137 (47.2), 124 (39.3).
UV-spectrum; λ max; H_2O (log ε) : 294 (3.54), 257 (4.14), 213 (4.26) nm.
IR-spectrum; υ max (KBr): 3300 cm^{-1}, 2920 cm^{-1}, 1520 cm^{-1}, 1250 cm^{-1}.

RESULTS AND DISCUSSION

ACCUMULATION OF CONIFERIN, CONIFERYL ALCOHOL AND 5-MPT

Suspension cultures of *Linum flavum* exhibited a rapid growth; cell dry weight increased from 7.4 to 19.5 g l^{-1} within 8 days. During the growth, the cells accumulated coniferin in enormous amounts. In Fig. 4, it can be seen that coniferin contents raised to over 12% on a dry weight basis in the stationary phase of the growth cycle. The rapid growth combined with the high contents, resulted in yields exceeding 2 g coniferin per l suspension culture (Fig. 4). Coniferin accumulation coincided with increases in fresh weight and packed cell volume. This finding, together with the high accumulation, indicates that the storage of coniferin is in the vacuoles of the cells. In general, the culture medium was completely free of coniferin, only in the late stationary phase negligible amounts were detected. 5-MPT was also accumulated and reached values of ca. 0.014% on a dry weight basis at day 11. For coniferyl alcohol maximal contents were found at day 7, ca. 0.004% on a dry weight basis.

Recently, coniferyl alcohol was found to stimulate the podophyllotoxin accumulation, as was demonstrated in feeding experiments performed with cell suspension cultures of *Podophyllum hexandrum* (Van Uden et al. 1990b; Chapter 5; Woerdenbag et al. 1990).

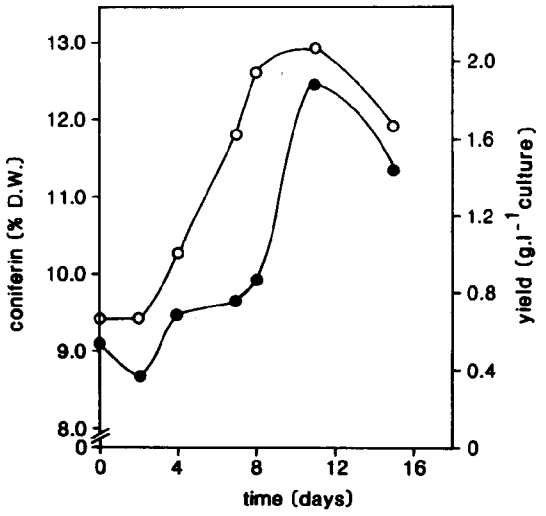

Fig. 4. Time course of coniferin contents (●) and coniferin yields (○) in suspension-grown cells of *L. flavum*.

5-MPT is strongly related to podophyllotoxin, therefore it was assumed that coniferyl alcohol could function as a precursor of 5-MPT as well. This is strengthened by our observation that the maximal 5-MPT content appeared 4 days after the maximal coniferyl alcohol content.

Coniferin contents in callus were only half of those in suspensions, an average value of 5.6% on a dry weight basis was found. Surprisingly, no coniferin could be detected in the leaves of *in vitro* grown plantlets, from which the callus and suspension cultures were initiated. Average coniferin levels in leaves, calli and suspensions are visualized in Fig. 5A. Many examples have been given from plant cell cultures producing little, if any, of the secondary metabolites accumulated in the intact plant (Zenk et al. 1977; Berlin 1988). In higher plants, secondary metabolites tend to accumulate in specific cell types at specific developmental stages (Balandrin et al. 1985; Rhodes 1985). Thus, it is to be expected that the production in undifferentiated cell cultures is very low or absent. However, more than 30 natural products are known to accumulate in cell cultures in levels exceeding those produced in the corresponding plants (Balandrin et al. 1988). In the present study this is demonstrated for coniferin.

In Fig. 5B and 5C the average 5-MPT and coniferyl alcohol levels in the plantlets and cell cultures at the moment of subculturing are compared. The highest accumulation of 5-

MPT occurred in leaf material, which had an average content of 0.100% on a dry weight basis. Callus contained only 0.035% and suspension-grown cells 0.014%.

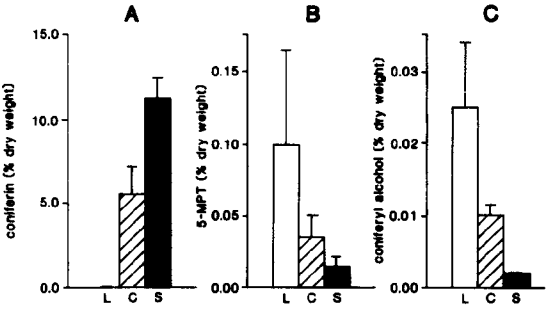

Fig. 5. Average values of coniferin (**A**), 5-methoxypodophyllotoxin (**B**) and coniferyl alcohol (**C**) contents in leaf (L), callus (C) and suspension (S) grown cells of *L. flavum* at the moment of subculturing (n=3, s.d. is indicated).

The levels of coniferyl alcohol were directly related to those of 5-MPT. This means that the highest percentage coniferyl alcohol, 0.025%, was found in the leaves, 0.010% in callus and the lowest amount, 0.002%, in suspension-grown cells. From the results dealing with metabolite production in leaves, callus and suspensions, it is concluded that the direct relationship between coniferyl alcohol and 5-MPT is in agreement with the suggestion that coniferyl alcohol is a precursor of 5-MPT.

PEROXIDASE AND β-GLUCOSIDASE ACTIVITIES

In order to gain more insight in the occurrence of the different levels of metabolites in leaves, callus and suspension-grown cultures of *L. flavum*, the activities of enzymes most directly involved in their biosynthesis and turnover, β-glucosidase and peroxidase, were determined (Fig. 2). β-Glucosidase converts coniferin into coniferyl alcohol, being an essential step in the biosynthesis of 5-MPT. Generally, glucosides of cinnamyl alcohols are not substrates for peroxidases, therefore aglycone formation is necessary (Freudenberg 1963, 1965; Hahlbrock 1977; Hösel and Todenhagen 1980). Peroxidase is an essential enzyme in the formation of the coniferyl alcohol radicals needed for further biosynthesis into lignans (Stöckigt and Klishies 1977; Jackson and Dewick 1984).
The activities of β-glucosidase and peroxidase during the growth cycle of *L. flavum* suspensions are shown in Fig. 6; generally, peroxidase activity was ca. 100 times higher than β-glucosidase activity.

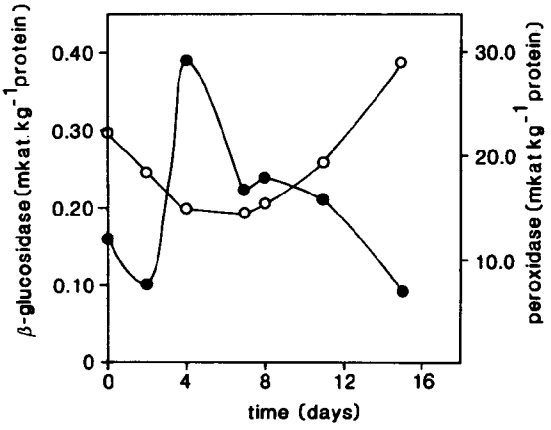

Fig. 6. Time course of β-glucosidase (●) and peroxidase (○) activity as found in the soluble protein fraction of suspension-grown cells of *L. flavum*.

β-Glucosidase activity increased to a maximal value of 0.4 mkat kg^{-1} protein at day 4 of the growth cycle and had already declined to lower levels before the stationary phase was reached. Fig. 7A shows a comparison of the β-glucosidase activities in the soluble protein fractions from leaf, callus and suspension cultures at the moment of subculturing. It can be seen that the activity in leaves of *in vitro* grown plantlets is 5-times higher than in callus and even 30-times higher than in suspension-grown cells. The ratios of β-glucosidase activities in soluble protein and pellet fractions were calculated to be: 6.4 ± 2.6, 4.8 ± 0.7, 4.9 ± 1.4 (n=3) for leaves, calli and suspensions, in the order given.

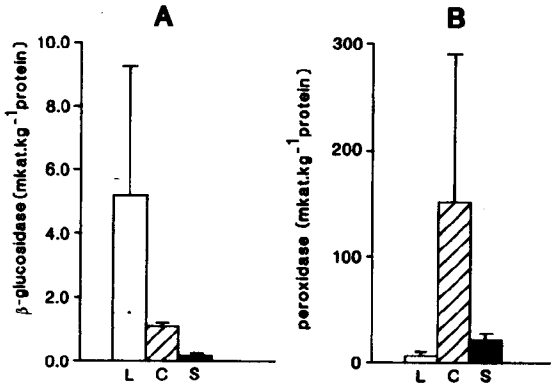

Fig. 7. Average values of β-glucosidase (**A**) and peroxidase (**B**) activity in the soluble protein fraction of leaf (L), callus (C) and suspension (S) grown cells of *L. flavum* at the moment of subculturing (n=3, s.d. is indicated).

Peroxidase activity started with declining until the stationary phase was reached, followed by an increase to values of ca. 30 mkat kg^{-1} protein. Peroxidase activities are shown in Fig. 7B, callus contained the highest activity (151 mkat kg^{-1} protein). Leaves contained

18-fold less activity and suspensions 7-fold less. No peroxidase activity at all was found in the pellet fraction of *L. flavum* cells.

ENZYME ACTIVITIES VERSUS METABOLITE ACCUMULATION

During the growth cycle, no clear relationship between β-glucosidase activity and coniferin contents in suspension-grown cells of *L. flavum* was found (Fig. 4, Fig. 6). However, the maximal activity at day 4 (Fig. 6) was for obvious reasons followed by a maximal coniferyl alcohol content at day 7. The β-glucosidase activities in leaves, calli and suspensions (Fig. 7A) showed a reciprocal relationship with coniferin contents (Fig. 5A) and are directly related to 5-MPT (Fig. 5B) and coniferyl alcohol (Fig. 5C) levels. Therefore, the fact that no coniferin was detected in leaf material might be due by the presence of high activities of β-glucosidase.

On the basis of these results, we suggest that one of the reasons for the high coniferin accumulation in suspension-grown cells of *L. flavum* is the low β-glucosidase activity in the soluble protein fraction. This suggestion also clarifies the direct relationship between the accumulation of coniferyl alcohol, 5-MPT and the β-glucosidase activity. For example, a low β-glucosidase activity leads to the conversion of relatively small amounts of coniferin into coniferyl alcohol, resulting in low coniferyl alcohol and high coniferin levels. Low amounts of the substrate coniferyl alcohol will then be dimerized to 5-MPT yielding in low contents of this lignan as well. On the other hand, the low 5-MPT contents might have been caused by the presence of low enzyme activities responsible for the further conversion of the aglucone coniferyl alcohol.

Activities of peroxidase were high compared with those of β-glucosidase in all investigated material. Since these high peroxidase activities were measured with a synthetic substrate, no direct conclusion can be drawn with respect to the rate of radical formation *in vivo*. Moreover, H_2O_2 may be a limiting substrate in the cinnamoyl alcohol conversion. In our opinion, maximal contents of 5-MPT as found in the stationary phase of the growth cycle were the direct result of higher substrate (coniferyl alcohol) levels rather than increased peroxidase activities. The observation that high peroxidase activity (151 mkat kg^{-1} protein) in callus tissue (Fig. 7B) did not correspond with increased 5-MPT levels (Fig. 5B), supports this opinion. Even the lower levels in leaves (8 mkat kg^{-1} protein) and suspensions (21 mkat kg^{-1} protein), were high enough to convert the present amounts of coniferyl alcohol into radicals, thus enabling 5-MPT synthesis.

THE ISOLATION OF CONIFERIN

Recently, it has been reported that coniferin stimulates the accumulation of podophyllotoxin (Van Uden et al. 1990b; Chapter 5; Woerdenbag et al. 1990). Since this

compound is not available commercially, we developed a procedure for the isolation of this compound from suspension-grown cells of *L. flavum* (Fig. 3). Lyophilized or fresh cells grown in 3 liter Erlenmeyer flasks were used. Coniferin levels of ca. 9% were still present in these cultures, although standard-grown cultures contained somewhat higher contents, ca. 11%. This decrease in metabolite content after upscaling cell cultures has also been reported for the production of rosmarinic acid by cell suspensions of *Coleus blumei*, demonstrating that this may be a more general problem (Zenk et al. 1977; Balandrin et al. 1985).

Fresh suspension cells proved to be the best starting material for the isolation of coniferin. When lyophilized cells were used, an optimal crystallization of crude coniferin was hindered, since a more viscous final fraction was obtained.

Coniferin was rapidly, within 30 min, and completely released when fresh cells were sonificated in 100% methanol. From the right-hand column in Fig. 3 it can be seen that most isolation steps gave small losses. The main problem was the crystallization of crude coniferin, probably caused by the presence of other disturbing components in this fraction. Crude coniferin was easily purified by recrystallization from water. Only a small percentage (<10%) of coniferin was lost during this proces. The isolated white crystalline coniferin was compared with authentic coniferin and showed identical UV, IR and mass spectra. The purity was 98% on a HPLC basis.

The developed procedure enabled a rapid and efficient isolation of coniferin. All organic solvents used could be recycled, as well as the resin XAD-4, making the process economically attractive. Discarded fractions which still contained coniferin were added to a new isolation procedure, in that way reducing coniferin losses to a minimum.

FINAL CONCLUSIONS

Suspension-grown cells of *Linum flavum* demonstrated a high capacity for coniferin accumulation. Within 11 days, 1.4 g nett of coniferin was produced by 1 l suspension.

Coniferin is not available commercially, while coniferyl alcohol is an expensive compound and easily obtained from coniferin. Directly derived products include vanillin, eugenol, *iso*-eugenol and zingerone. Coniferin can stimulate the production of podophyllotoxin. Therefore, the high-producing capacity, together with an efficient isolation procedure may be of commercial and scientific interest. The observed coniferin levels fulfil the requirements for economical production of secondary metabolites, as has been described in cost analyses (Balandrin et al. 1985; Berlin 1988), on the condition that the market for the above-mentioned products is sufficiently large. The enormous accumulation of coniferin by cell suspensions of *L. flavum* provides an exciting research challenge, for example to study lignin and lignan biosynthesis.

ACKNOWLEDGEMENTS

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CHAPTER 7

IMPROVEMENT OF THE PRODUCTION OF 5-METHOXY-PODOPHYLLOTOXIN USING A NEW SELECTED ROOT CULTURE OF *LINUM FLAVUM* L.

(Published in Plant Cell Tissue and Organ Culture 27: 115-121)

ABSTRACT

A new, morphologically root-like cell line of *Linum flavum* was selected and used for the optimization of the production of 5-methoxypodophyllotoxin (5-MPT). The omittance of naphthaleneacetic acid from the medium resulted in further root formation, which was accompanied with a 2.6 fold increase of 5-MPT levels. The feeding of the phenylpropanoids L-tyrosine and L-phenylalanine resulted in enhanced 5-MPT contents. After the removal of vitamins together with the phytohormone from the medium, 5-MPT levels were enhanced 6 times. The maximal content ever found using this production medium was 1.01% on a dry weight basis. The application of higher sucrose concentrations led to proportional biomass increase, cells grown on 6% sucrose yielding 121.4 mg l^{-1} 5-MPT.

INTRODUCTION

Podophyllotoxin is a naturally occurring lignan, which is extracted from the rhizomes of *Podophyllum peltatum* and *Podophyllum hexandrum* (Berberidaceae) and serves as a starting compound for the preparation of semi-synthetic derivatives. Since 1950, podophyllotoxin and many related compounds from *Podophyllum* have been clinically tested for cytotoxic properties. Thus far, only two compounds, teniposide (VM-26) and etoposide (VP-16-213), have been found suitable for human use and are now applied in the anti-tumour therapy (Issell et al. 1984).

The search for new cytostatics is still continuing, because of severe side effects of the applied anti-tumour agents and because of the insensitivity of several neoplastic malignancies for these chemotherapeuticals (Saito et al. 1986a, 1986b; Thurston et al. 1986; Clark and Slevin 1987; Stringfellow and Schurig 1987; Green 1989).

Stähelin and Von Wartburg (1989) reported on the problem of finding *Podophyllum* compounds of higher clinical utility. An additional problem, which may arise in the future is that *P. hexandrum* becomes a threatened species, caused by its intense collection and the lack of organized cultivation (Gupta and Sethi 1983). As a reaction to these poor prospectives, the production of podophyllotoxin by means of biotechnological techniques may form an alternative. The multiplication of *Podophyllum* species by means of somatic embryogenesis and plant regeneration has gained much interest (Chuang and Chang

1987a, 1987b; Arumugam and Bhojwani 1989). Recently, several reports on the biosynthesis of podophyllotoxin and related lignans by plant cell cultures have appeared (Kadkade 1981, 1982; Berlin et al. 1986, 1988; Van Uden et al. 1989; 1990a, 1990b, 1990c, 1991; Chapters 2, 3, 4, 5, 6; Woerdenbag 1990; Wichers et al. 1990; Hyenga 1990). A number of these studies are dealing with 5-methoxypodophyllotoxin (5-MPT) produced by cell cultures initiated from *Linum flavum*.

5-MPT (Chapter 6, Fig. 1) is a relatively new discovered compound and its presence has been demonstrated in several *Linum* species and in *Juniperus sabina* as well (Berlin et al. 1986, 1988; Broomhead and Dewick 1990; San Feliciano et al. 1990; Van Uden et al. 1990a, 1991; Chapters 3, 5; Wichers et al. 1990). Based on the structural resemblance with podophyllotoxin, 5-MPT may be an interesting starting compound for the preparation of new semi-synthetic derivatives with anti-tumour properties.

MATERIALS AND METHODS

PLANT MATERIAL AND CULTURE CONDITIONS

Plantlets and callus of *Linum flavum* L. (Linaceae) were obtained and grown as described recently (Van Uden et al. 1990a; Chapter 3). The root-like culture used in this study was obtained by selecting organized root tissue, which occasionally developed on dark-grown callus. After selecting and subculturing for a half-year period, a callus culture predominantly consisting of roots was obtained. A shake culture of these roots has been maintained in our laboratory for more than two years. These cultures are subcultured on the standard medium, containing MS-salts (Murashige and Skoog 1962) and B5-vitamins (Gamborg et al. 1968) supplemented with 3% (w/v) sucrose, 0.88 mg l^{-1} folic acid, 2.0 mg l^{-1} glycine and 3.0 mg l^{-1} naphthaleneacetic acid (NAA). The root-like cultures were incubated on a rotary shaker (120 rpm) at 26 °C in the dark and subcultured by adding 100 ml of a two-week-old root culture to 200 ml of fresh medium. Culture samples of ca. 10 ml were taken during the growth cycle.

OPTIMIZATION EXPERIMENTS

The optimization experiments were performed under the standard culture conditions, only the medium composition was changed. In the different production media used, the phenylpropanoids L-phenylalanine, L-tyrosine or *trans*-ferulic acid were added in concentrations of 1.0, 3.0 and 5.0 mM, before autoclavation. Sucrose was used in concentrations of 3, 6 or 9% (w/v). In all experiments, sampling of the differentiated cultures during the growth cycle was done by aseptically taking some root-like material with a pair of pincers. Usually, after 14 days the remainder of the cultures was harvested and dried. In experiments where subculturing of this highly differentiated tissue was necessary, ca. 30% of the material was transferred to a flask containing 200 ml of fresh medium.

EXTRACTION PROCEDURE

Samples were dried by lyophilization and powdered in a mortar. Crude extracts to be used for HPLC analysis were prepared according to a described procedure (Van Uden et al. 1989, 1991; Chapters 2, 6).

ANALYSIS BY MEANS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

5-MPT and coniferin were analyzed using a HPLC-UV system as applied in previous studies (Van Uden et al. 1989, 1991; Chapters 2, 6). The production rates were calculated as described in Chapter 2.

RESULTS AND DISCUSSION

SELECTION OF A ROOT-LIKE CULTURE

The undifferentiated cell line of *L. flavum*, which has been involved in our investigations so far (Van Uden et al. 1990a; Chapter 3), sometimes spontaneously developed some organized tissue such as roots or shoots. Callus cultures growing under light conditions mostly regenerated shoots, whereas callus grown in the dark mainly showed root formation.

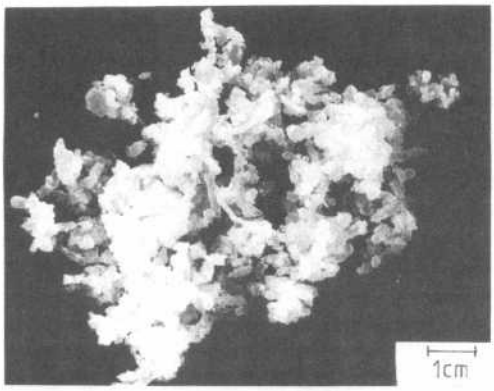

Fig. 1A. Morphological structure of the root-like culture of *L. flavum* grown in standard medium, *i.e.* including 3 mg l^{-1} NAA.

By subculturing only the roots or root-like tissues for a period of half-a-year, a callus culture with a strong tendency for adventitious root development could be selected. The roots were coloured white-yellowish, very small (ca. 5 mm) and embryo-like. Therefore, we preferably describe this culture as root-like and not as a genuine root culture. After the transfer of this root regenerating callus to liquid medium the root formation was not affected. Remarkably, after growing in liquid medium over a period of more than two years, the ratio roots/cell aggregates was in favour of root formation. These cells of *L. flavum* apparently give preference to the morphological state of roots, even when 3.0 mg l^{-1} NAA, normally promoting undifferentiated growth, is present. The root-like character of the cultures growing in liquid medium is also embryo-like, which is shown in Fig. 1A.

Recently, we reported that the transfer of fully undifferentiated cells of *L. flavum* to hormone-free medium resulted in a giant enhancement of the 5-MPT content, namely from 0.015 to 0.73% on a dry weight basis (Van Uden et al. 1990a; Chapter 3). In this study, a comparable experiment was carried out, using the new root-like cell line. The standard-growing root-like culture of *L. flavum* generally contained 0.10 ± 0.06% (n=8) 5-MPT on a dry weight basis.

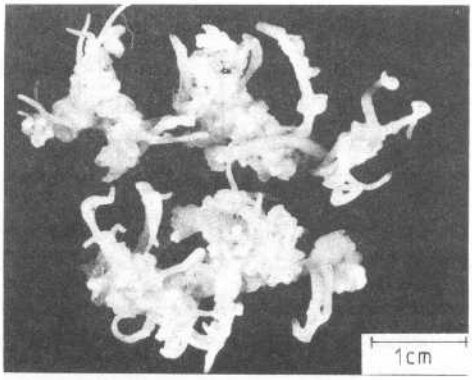

Fig. 1B. Morphological structure of the root-like culture of *L. flavum* grown in standard medium without NAA.

After the transfer to NAA-free medium, roots developed rapidly. The length never exceeded 10 mm, the morphology is visualized in Fig. 1B.

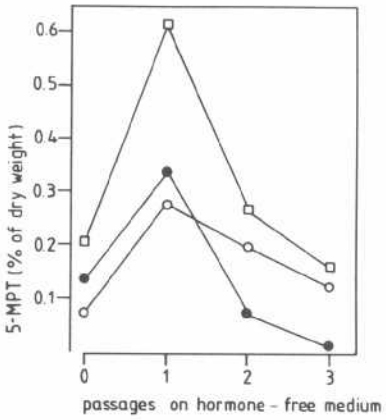

Fig. 2. Course of the accumulation of 5-MPT in a root-like culture of *L. flavum* grown in liquid medium, as aglucone (●), as β-D-glucoside (○) and the total of both (□), during three passages on hormone-free medium.

Under the phytohormone lacking conditions, the 5-MPT content increased from 0.13 to 0.34% during a growth period of 14 days (Fig. 2), the dry weight increased from 4 to 10 g l^{-1} within this period. At the same time, the amount of 5-MPT present as β-D-glucoside raised from 0.07 to 0.28%, meaning a 4-fold enhancement. Thus, the absolute content of

5-MPT present in the cells after 14 days of growing in a NAA-free medium was 0.62% on a dry weight basis. After enzymatic hydrolysis of the extracts in order to determine the amount of 5-MPT present as β-D-glucoside, HPLC analysis revealed the presence of many unknown glucosidic compounds. Since the ultimate aim of the study was to obtain pure 5-MPT, we decided to isolate 5-MPT from the less complex fractions, that contain naturally occurring aglucones, and not from the hydrolyzed extracts. Therefore in further optimization experiments we paid attention to the presence of 5-MPT as an aglucone only. After following the production of 5-MPT during the first passage on hormone-free medium, it was found that highest contents of this lignan are present after 14 days. Subculturing of the root-like tissue for two more growth cycles on a medium lacking phytohormone was coupled with a decrease of 5-MPT contents, as depicted in Fig. 2, and with a reduced capacity of forming biomass. It seems that after the omittance of NAA, the cells can rapidly differentiate, form biomass and produce higher levels of a secondary metabolite, but only during a very limited time. Therefore, such a medium may be considered as a production medium and can not be used for maintenance of this cell line. Since the results with respect to the 5-MPT contents were rather promising, a number of production media were tested. The development of better production media was based on four approaches: **1.** Increase in content of 5-MPT by addition of the phenylpropanoids L-tyrosine, L-phenylalanine or *trans*-ferulic acid. **2.** The simplification of the media by omittance of vitamins, salts or hormone. **3.** Increase in biomass, possibly leading to higher yields of 5-MPT by using higher sucrose concentrations. **4.** Development of media based on the results of the approaches **1**, **2** and **3**.

1. ADDITION OF PHENYLPROPANOIDS

The first experiments after addition of the phenylpropanoids revealed that the concentration should not exceed 3 mM. At higher concentrations, growth was negatively affected, indicating toxicity. Ferulic acid was not suitable at all, since no 5-MPT enhancement was found and growth ceased at all concentrations used. The addition of L-tyrosine or L-phenylalanine to NAA-containing as well as NAA-lacking cultures, generally raised the endogenously accumulated 5-MPT levels. 5-MPT contents were enhanced a factor 1.3 to 3.3, compared with the contents of roots that grew without phenylpropanoids. The maximal yield was obtained after the addition of 1 mM L-phenylalanine to the standard medium, being 29.6 mg l^{-1}.

Precursor experiments with *Linum flavum* root cultures using ^{14}C-labelled phenylalanine have been performed by Berlin et al. (1988) and did not result in a distinct increase of 5-MPT. However, L-phenylalanine has been reported previously to be a precursor of podophyllotoxin (Ayres et al. 1981; Jackson and Dewick 1984). The 5-MPT

enhancements found in our study can not with certainty be ascribed to bioconversion, since no labelled precursors were used. The added phenylpropanoids could also have induced stress, both factors which possibly explain the increased 5-MPT contents.

2. SIMPLIFICATION OF THE MEDIUM

The most remarkable step performed in optimizing a production medium was the removal of vitamins. Culturing the root-like tissue for 14 days on a medium lacking vitamins as well as hormone, resulted in an increase of the 5-MPT levels. Generally, the 5-MPT levels were enhanced 3-10 times compared with cultures growing on the standard medium. The increase resulted in the highest 5-MPT content ever measured (aglucone only), 1.01% on a dry weight basis, corresponding with a production rate of 8.4 mg l^{-1} day^{-1}. Generally, the dry weight increased from 4 to 12 g l^{-1} within a growth period of 14 days.
Thus far, no theory has been found to explain this phenomenon, although the effect of most production media is that they impose a stress on the cells (Berlin 1988). In an attempt to extremely simplify the medium, cells were grown on a solution of sucrose only. Biomass production was unaffected, but unfortunately the 5-MPT contents decreased rapidly.

3. THE INCREASE OF BIOMASS

The increase of biomass produced in root-like cultures on different sucrose concentrations, was almost proportional. Sucrose concentrations of 3, 6 and 9% yielded dry weights of 10, 24 and 34 g l^{-1} respectively, determined after a growth period of 14 days. The 5-MPT levels were lower when grown on concentrations of 6 and 9% sucrose compared with 3%, losses of ca. 25 and 60% were found respectively. With respect to the overall yield, a production medium containing 6% sucrose was found to be optimal. It has been suggested by Berlin (1988) that the increases of dry mass in production media with high levels of sucrose are probably due to an enhanced storage or incorporation of the carbon-source and not to cell division and growth.

4. COMBINATIONS

On the basis of the above results, a number of medium compositions were examined. These combinations and the obtained results, including all production rates are summarized in Table 2. The highest percentage of 5-MPT present as an aglucone was found in cultures grown on a medium with only MS-salts supplemented with 1 mM L-tyrosine, namely 0.71%, while non-fed cultures contained 0.68% 5-MPT on a dry weight basis. Generally, 5-MPT yields were maximal on media consisting of MS-salts supplemented with 6% sucrose, with, or without added phenylpropanoid. The highest

yield found in these experiments was 128.7 mg l^{-1} (rate of production: 8.9 mg 5-MPT l^{-1} day^{-1}), meaning a 6.6 fold increase as compared with the standard yield of 19.6 mg l^{-1}. On the other hand, the ratio was only 2.8 when calculated on percentages of dry weight, emphasizing the importance of using high carbon-source concentrations for production purposes. The presence of L-tyrosine or L-phenylalanine in the production media given in Table 1 did not lead to the 5-MPT enhancements as described under **1.** ADDITION OF PHENYLPROPANOIDS. Apparently, when the production of 5-MPT was already strongly stimulated, in the absence of vitamins, no further increase could be achieved by feeding of these phenylpropanoids.

Recently, it has been reported that low 5-MPT levels correspond with high coniferin levels, a phenylpropane glucoside naturally occurring in our *Linum* cell cultures (Van Uden et al. 1991). Therefore, this relationship was examined in the newly selected root-like culture grown in the various production media. Except for cultures grown on enhanced sugar concentrations, the reciprocal relationship between 5-MPT and coniferin could be confirmed in these experiments. No unambiguous relationship could be estabished between the coniferin and 5-MPT contents in the cultures grown on 6% sucrose. Since the coniferin accumulation is normally stimulated upon the use of higher sucrose levels (unpublished results) the finding of the highest coniferin content (7.37%) in cultures grown in a medium, which has been supplemented with 9% sucrose, and with a relatively low 5-MPT content, was not unexpected.

FINAL CONCLUSIONS

Using the optimization approaches, several media have been developed, which proved to be suitable for the production of 5-MPT. The simple medium, solely consisting of MS-salts supplemented with 6% sucrose, combined with the root-like culture of *L. flavum* has been chosen for scale-up experiments. In order to produce 5-MPT using cell cultures of *L. flavum,* induction of differentiation of the cell tissue is needed. The next step will be to show whether biotechnologically produced 5-MPT may be a serious candidate to compete with podophyllotoxin as a potential substrate for the chemical synthesis of anti-tumour agents. In the following chapter, the isolation of 5-MPT from the root-like cultures of *L. flavum* L., its purification and finally, the determination of the cytotoxicity are presented.

Table 1. Effects of various media on the production of 5-MPT and coniferin using a root-like culture of *L. flavum*.

Medium	5-MPT (% of dry weight)	Dry weight ($g\ l^{-1}$)	Yield ($mg\ l^{-1}$)	Rate of production ($mg\ l^{-1}\ day^{-1}$)	Coniferin (% of dry weight)
Standard	0.20	9.8	19.6	1.1	6.17
Standard-NAA-Vit[1]	0.68	12.6	85.7	5.8	4.23
Standard-NAA-Vit+1mM PA[2]	0.60	12.1	72.6	4.9	3.94
Standard-NAA-Vit+1mM TYR[3]	0.71	12.4	88.6	6.0	4.57
Standard-NAA-Vit with 6% sucrose	0.51	23.8	121.4	8.4	4.80
Standard-NAA-Vit+1mM PA with 6% sucrose	0.55	23.4	128.7	8.9	6.86
Standard-NAA-Vit+1mM TYR with 6% sucrose	0.50	24.3	121.5	8.4	6.46
Standard-NAA-Vit with 9% sucrose	0.25	34.3	85.8	5.9	7.37

1 Vitamins
2 L-phenylalanine
3 L-tyrosine

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CHAPTER 8

ISOLATION, PURIFICATION, AND CYTOTOXICITY OF 5-METHOXYPODOPHYLLOTOXIN, A LIGNAN FROM A ROOT CULTURE OF *LINUM FLAVUM* L.

(Journal of Natural Products 55: 102-110)

ABSTRACT

A procedure has been developed for the isolation of 5-methoxypodophyllotoxin (5-MPT) from a high-producing root-like culture derived from *Linum flavum* L.. A closely related lignan, 5'-demethoxy-5-methoxypodophyllotoxin, was also present in the root-like culture and was the main cause for problems in isolating pure 5-MPT. Important steps in the isolation procedure are: dichloromethane extraction, XAD-4 adsorption and XAD-8 column chromatography followed by silica gel chromatography, using two different mobile phases. The isolated 5-MPT was for more than 99% pure and possessed the desired stereochemical configuration, namely (-)-5-MPT. The *in vitro* cytotoxicity of the isolated 5-MPT against EAT and HeLa cells, was determined and compared with those of 5-MPT-4-β-D-glucoside, podophyllotoxin, etoposide and teniposide. It appeared that 5-MPT has comparable cytotoxic potency as podophyllotoxin.

INTRODUCTION

Etoposide (VP-16-213) and teniposide (VM-26) are two clinically applied semi-synthetic cytostatics, chemically prepared from the natural occurring podophyllotoxin (Clark and Slevin 1987; Holthuis 1988; Stähelin and Von Wartburg 1989). The total chemical synthesis of podophyllotoxin is very complicated, mainly because of the presence of four chiral centers, a rigid *trans*-lactone and an axially locked 1-aryl substituent (Forsey et al. 1989). Therefore, podophyllotoxin is still extracted from the rhizomes of *Podophyllum* species (Berberidaceae)(Issell et al. 1984). However, the supply of *P. hexandrum* plants, which contain about 4.3% podophyllotoxin on a dry weight basis, has become limited, due to both intensive collection and lack of organized cultivation (Gupta and Sethi 1983). As a result, high costs have to be spended in obtaining podophyllotoxin from the plant rhizomes. Therefore, the production of podophyllotoxin and related lignans by means of biotechnological procedures has been considered as an attractive alternative. During the last decade, a series of reports on the biosynthesis of podophyllotoxin and related compounds using plant cell cultures has been published (Kadkade 1981, 1982; Berlin et al. 1986, 1988; Van Uden et al. 1989, 1990a, 1990b, 1990c, 1991a, 1991b; Chapters 2, 3, 4, 5, 6, 7; Hyenga et al. 1990; Woerdenbag et al. 1990; Wichers 1990, 1991). Several reports have dealt with the production of 5-methoxypodophyllotoxin (5-MPT) by cell

cultures from *Linum flavum*. So far, the presence of 5-MPT has only been demonstrated in a few *Linum* species and in *Juniperus sabina* (Broomhead and Dewick 1990; San Feliciano et al. 1990). Recently, we selected a root-like culture of *L. flavum* and developed a medium for an optimal production of 5-MPT (Van Uden et al. 1991a; Chapter 7).
In the present study, the isolation and purification procedure for 5-MPT from these roots is described.

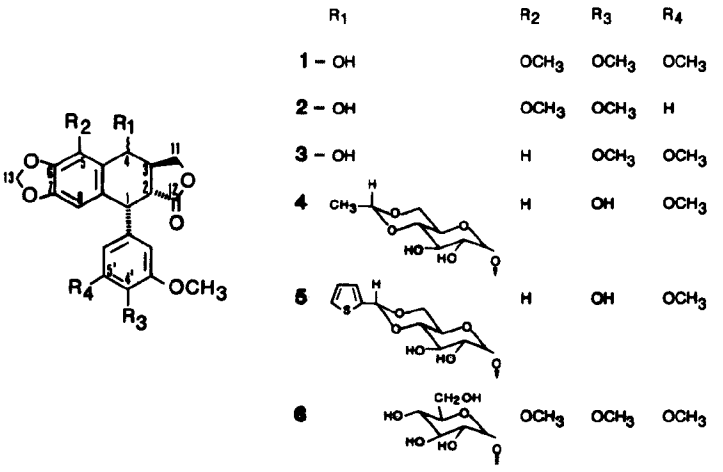

Fig. 1. The chemical structure of 5-methoxypodophyllotoxin (**1**), 5'-demethoxy-5-methoxypodophyllotoxin (**2**), podophyllotoxin (**3**), etoposide (**4**), teniposide (**5**) and 5-methoxypodophyllotoxin-4-β-D-glucoside (**6**).

The search for new cytostatics is still in progress because of the severe side effects of the generally used anti-tumour agents and because of the insensitivity of many neoplastic malignancies for the applied therapeuticals (Stringfellow and Schurig 1987; Clark and Slevin 1989; Green 1989; Stähelin and Von Wartburg 1989; Ayres and Loike 1990). Based on the chemical resemblance with podophyllotoxin, 5-MPT may be an interesting starting compound for the preparation of new semi-synthetic derivatives with anti-tumour properties. Therefore, in this study, the cytotoxic activity of the isolated 5-MPT was tested and compared with authentic podophyllotoxin, 5-MPT-4-β-D-glucoside, etoposide and teniposide. The chemical structures of these compounds are depicted in Fig. 1.

MATERIALS AND METHODS

CULTURE CONDITIONS

A root-like cell line of *Linum flavum* L. (Linaceae) was obtained by selection and grown as described recently (Van Uden et al. 1991b; Chapter 7). In this study, the cell material used for the isolation of 5-MPT was grown on a production medium consisting of MS-salts (Flow Laboratories) (Murashige and Skoog 1962), 6% sucrose, no phytohormone and no vitamins (Van Uden et al. 1991b; Chapter 7). Root-like structures rapidly developed and after growing for two weeks on this medium, the cells were harvested by suction filtration. The 5-MPT content generally ranged from 0.35 to 0.68%, on a dry weight basis.

EXTRACTION AND ISOLATION PROCEDURE

The root-like tissue was dried by lyophilization and subsequently powdered in a mortar. The isolation of 5-MPT was performed according to the scheme given in Fig. 2. For sonification of the cells in methanol, 100 g powdered cells were incubated with ca. 0.8 l of 80% methanol in a sonification bath (Branson).
XAD-column chromatography for crude 5-MPT was an important step in the isolation procedure. For optimization, several Serdolit® XAD-adsorbent resins were applied. The XAD-2, -4, -7 and -8 adsorbent resins (300-1000 µm; research grade) were all purchased from Serva and regenerated by shaking with water, methanol and dichloromethane, respectively. Small-scale optimization experiments with the XAD-resins were performed using 10 ml syringes packed with 10 ml XAD-resin. Ca. 30 mg samples of 5-MPT, contaminated with 5'-demethoxy-5-MPT (absorbance ratio at UV-290 nm is ca. 14 in favour of 5-MPT), were eluted with 80% methanol.
The XAD-8 column as used in the larger scale isolation procedure consisted of a glass tube (60 cm x 16 mm i.d.) and was packed with 102 ml XAD-8 resin. The flow of the eluent was 2 ml min^{-1}, and the first four 100 ml fractions were collected.
Subsequently, two columns packed with silica gel (Baker I.T., 70242, average particle size 40 µm) were prepared. The column, which was eluted with chloroform/aceton, (50 cm x 16 mm i.d.) was packed with 78 ml silica. Fourteen fractions of 10 ml were collected. From the column eluted with n-heptane/dichlomethane/methanol, (60 cm x 32 mm i.d.) containing 376 ml silica gel, 102 fractions of 50 ml were collected.
In the isolation scheme of Fig. 2, the addition of water has been mentioned in several purification steps. Generally, twice the volume of the methanol fraction was added for dilution. When extraction with dichloromethane has been indicated, this solvent was added in a volume equal to the methanol fraction.

ANALYSIS BY MEANS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

All isolation steps were controlled using HPLC with UV-detection at 290 nm as described previously (Van Uden et al. 1990a; Chapter 3). As a reference solution, 0.1 mg ml^{-1} of authentic 5-MPT in methanol was used. For the identification of 5'-demethoxy-5-MPT, a methanolic solution of unknown concentration was used. Both references were a kind gift from TNO, Zeist, The Netherlands.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

A cell extract of *Linum flavum* prepared as desribed previously (Van Uden et al. 1991a; Chapter 7), as well as a methanolic reference solution of 5'-demethoxy-5-MPT were subjected to GC-MS.
Gas chromatography coupled with mass spectrometry was performed on a Finnigan 3300 GC-MS system under the following conditions: column: fused silica CP-Sil 5CB, 25 m x

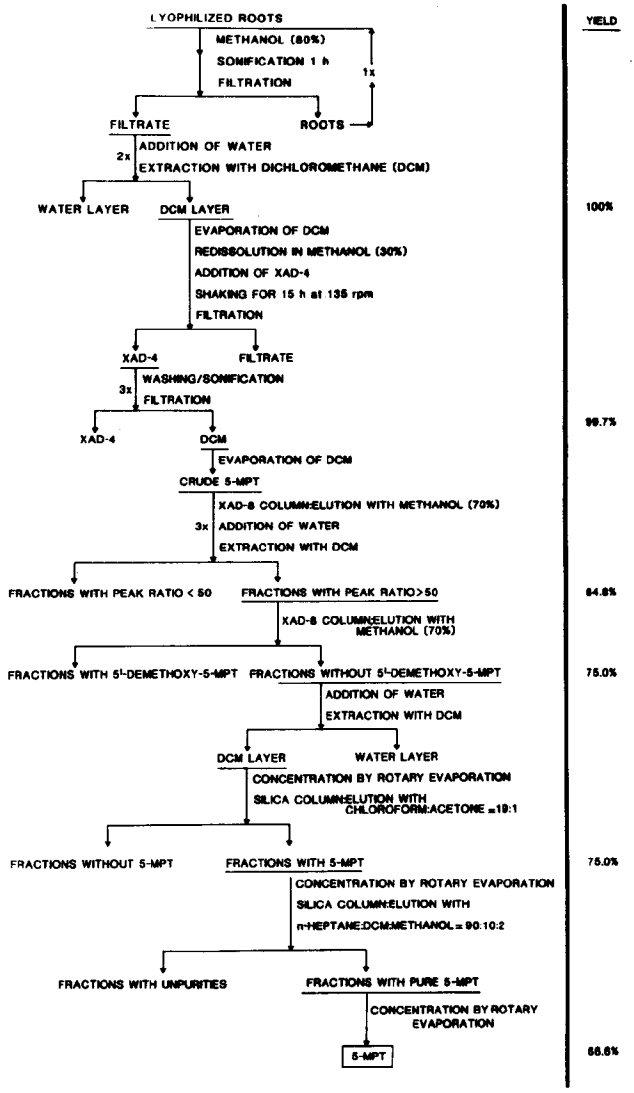

Fig. 2. Scheme for the isolation of 5-MPT from a root-like culture of *L. flavum*. In the right column the 5-MPT yields are indicated as a percentage of the initial amount.

0.33 mm i.d. (Chrompack); temperature program: 200-295 °C, 6 °C min^{-1}; temperature injection system: 250 °C; carrier gas helium 1 ml min^{-1}; injected volume: 1 µl.
Mass identification was performed using electron impact at an ionization energy of 70 eV; temperature ion source: 250 °C. Cycling time during the acquisition of the mass spectra was 1 s. 5'-demethoxy-5-MPT: [M^+] 414 (100), 258 (30), 231 (23), 151 (56), 139 (98).
The isolated and purified 5-MPT was directly introduced into the mass spectrometer by means of the dried insertion probe; m/z, relative intensities (%): 5-MPT: [M^+] 444 (100), 258 (14), 219 (16), 181 (21), 168 (79), 153 (21).

ULTRAVIOLET (UV) AND INFRARED (IR) SPECTROSCOPY

UV-spectra of 5-MPT were recorded on a Shimadzu spectrophotometer UV-160 and IR-spectra (KBr-discs) on a Beckman Acculab 2 infrared spectrophotometer.
UV-spectrum; λ max; methanol (log ε): 219 (5.54), 279 (3.38) nm.
IR-spectrum; υ max in cm^{-1} (KBr): 3560, 2960, 2900, 2840, 1785, 1620, 1595, 1505, 1480, 1250, 1125, 1095, 1000, 940, 845.

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

^1H-and ^{13}C-NMR (Attached Proton Test) spectra of 5-MPT were recorded on a Varian VXR-300 system. Purified 5-MPT was dissolved in deuterated chloroform.
^1H-NMR (300 MHz) δ 6.44 (2H, s, H-2', H-6'), 6.30 (1H, s, H-8), 5.95 (2H, s, H-13a, H-13b), 5.03 (1H, d, H-4, J_{4-3} 8.1), 4.64 (1H, dd, H-11a, J_{11a-3} 7.1, $J_{11a-11b}$ 8.8), 4.54 (1H, d, H-1, J_{1-2} 4.4), 4.16 (3H, s, 5-OCH_3), 4.07 (1H, dd, H-11b, J_{11b-3} 10.6), 3.81 (3H, s, 4'-OCH_3), 3.77 (6H, s, 3'-OCH_3, 5'-OCH_3), 2.75 (1H, dd, H-2, J_{2-3} 14.8, J_{2-1} 4.4), 2.87 (1H, m, H-3).
^{13}C-NMR (75.4 MHz) δ 174.24 (C-12), 152.41 (C-3'+C-5'), 149.27 (C-7), 141.40 (C-5), 136.90 (C-6), 134.76 (C-1'), 134.54 (C-4'), 132.64 (C-9), 124.76 (C-10), 107.93 (C-2'+C-6'), 104.18 (C-8), 101.22 (C-13), 71.77 (C-11), 70.35 (C-4), 60.81 (C-4'-OCH_3), 59.79 (C-5-OCH_3), 56.03 (C-3'-OCH_3 + C-5'-OCH_3), 44.95 (C-2), 44.42 (C-1), 38.88 (C-3).

POLARIMETRY

Specific optical rotations of 5-MPT were determined using a Perkin-Elmer 241 polarimeter equipped with 10 cm cuvettes.
$[\alpha]^{20}$; (λ in nm): -129.5° (589.3), -136.5° (578), -156.3° (546), -284.4° (436), -494.9° (365).
(c = 0.99 in chloroform).

ELEMENTAL ANALYSIS

5-MPT, $C_{23}H_{24}O_9$, calculated: C = 62.16, H = 5.44; found: C = 62.39, H = 5.81

MELTING POINT

The melting point of 5-MPT was determined on a Büchi apparatus and is uncorrected.
m.p.: 95-100 °C; effervescence.

CYTOTOXICITY TEST

The cytotoxicity of the lignans was tested against the murine Ehrlich ascites tumour (EAT) and the human HeLa (cervix uteri) cell lines. These cell lines were cultured routinely at 37 °C, in a humidified incubator with 5% CO_2 at the Department of Radiobiology, University of Groningen, The Netherlands. EAT cells were grown in suspension culture in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (Gibco) plus 50 μg ml^{-1} streptomycin and 50 IU ml^{-1} penicillin G. HeLa cells were cultured in Joklik's modification of MEM (Gibco) with 10% fetal calf serum, 50 μg ml^{-1} streptomycin and 50 IU ml^{-1} penicillin G. The doubling time of the EAT cells was 12-14 h and of the HeLa cells approx. 24 h. The experiments were performed with exponentially growing cells. The viability of the cells used in the experiments exceeded 95%, as determined with trypan blue. The lignans, being: 5-MPT, podophyllotoxin (Sigma), etoposide (Bristol-Myers), teniposide (Bristol-Myers, via Dept. of Radiobiology, Groningen, The Netherlands) and 5-MPT-4-β-D-glucoside (TNO, Zeist, The Netherlands), were dissolved in 96% ethanol just before use at a concentration of 0.5 or 5 mM, depending on the solubility of the compounds. In addition, a dilution containing 75% of the former solutions was prepared. In the experiments, microtiter test plates (Cel-Cult, Sterlin Ltd., Feltham, UK) with eight rows of 12 concave-bottomed wells each, were used. In these wells, 24 two-fold dilutions of the lignans were made in 100 μl culture medium. Subsequently, 2000 viable cells in 100 μl medium were added. The test plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for 4 days. The cytotoxic effect of the lignans was then visually determined by comparing the diameter of the sedimented cell pellets. The ED_{50} values, defined as the drug concentration causing 50% growth inhibition of the tumour cells, were calculated. As a regular control in the experiments, the growth of the tumour cells without addition of the cytotoxic agent was checked.

RESULTS AND DISCUSSION

EXTRACTION AND ISOLATION PROCEDURE

The extraction of lyophilized root-like cell material by means of sonification in 80% methanol for 1 h was the first step in the isolation procedure (Fig. 2). This extraction method proved to be very suitable for small samples, usually consisting of 100 mg quantities of dried cell material (Van Uden et al. 1989, 1990a, 1990b, 1990c, 1991a, 1991b; Chapters 2, 3, 4, 5, 6, 7; Woerdenbag et al. 1990). The use of larger quantities resulted in a strongly reduced efficiency of the extraction. When more than 100 g of dried cell material was used, the efficiency was lowered by 50-70%. Possibly, the applied energy is insufficient to damage the cells, when high biomass concentrations are present. Therefore, the extraction procedure still has to be optimized.

An emulsion sometimes occurred during the extraction of the polar water/methanol phase with dichloromethane. The addition of one or two drops of concentrated hydrochloric acid was sufficient to clarify the mixture, in order to displace the 5-MPT to the latter apolar phase. Care was taken not to let the pH of the methanol/water layer drop below 2. At a pH of 1, HPLC-analysis revealed a partial decomposition of 5-MPT into unknown compounds. The use of base had to be avoided at any case, since this led to dramatic

5-MPT losses. Strong decomposition of 5-MPT was also observed when a solution in water/methanol was heated above 40 °C, although 5-MPT in pure methanol could be heated or evaporated without decomposition of the lignan. As a consequence, water/methanol fractions, which were collected from the XAD-8 column, could not be concentrated by rotary evaporation. This problem was solved by adding an extra amount of water to these fractions first, followed by extraction with dichloromethane and subsequent evaporation of this apolar phase.

Decomposition of podophyllotoxin in hot 96% ethanol has been described in detail by Buchardt et al. (1986), while the sensitivity of podophyllotoxin to acids and bases has long been recognized. For example, even weak bases like ammonia and sodium acetate rapidly induced epimerization leading to picropodophyllin (Hartwell and Schrecker 1958; Ayres and Loike 1990).

As a result of the strong structural resemblance, a similar decomposition pattern for 5-MPT is likely valid. More generally, working with 5-MPT should always be accompanied with studying the inexhaustible amount of publications on podophyllotoxin.

The use of XAD-4 was introduced to adsorb 5-MPT, while polar impurities like dyes, proteins, sugars and salts remained behind in the mobile 30% methanolic phase.

Crude 5-MPT, as mentioned in the isolation scheme of Fig. 2, was yellowish. The main impurity consisted of an HPLC-UV detectable compound with a retention time of 1 min less than the retention time of 5-MPT, which is approx. 10 min. When the HPLC-UV chromatogram peak of the unknown compound was compared with a reference 5'-demethoxy-5-MPT, the retention times were identical. The occurrence of this lignan in *L. flavum* plants and suspension cultures has been reported very recently by Wichers et al. (1991). Further analysis of the unknown compound in our material by means of combined GC-MS and comparison of the MS-data with those of the reference, confirmed that the unknown compound was 5'-demethoxy-5-MPT. Based on the very strong chemical resemblance, serious problems in the separation of 5'-demethoxy-5-MPT and 5-MPT were expected. During several steps of the isolation procedure, the two lignans remained unseparable indeed, indicating almost identical physical properties, such as polarity and solubility. To investigate the possibilities of separating these closely related compounds, we chose to examine a number of XAD adsorbent resins.

After eluting the mixture on 10 ml XAD-7 or XAD-8 with 50 ml 80% methanol, 100% of the applied 5-MPT was recovered. For comparison, using XAD-2 and XAD-4, only 33% and 50% were recovered, respectively. Except for a rapid recovery of the applied sample, the separation ability of the resin is important. From Fig. 3 it can be seen that from the four tested XAD-resins, XAD-8 met these requirements the best. Practically, this means that the first fractions collected from an XAD-8 column are enriched with 5-MPT and

contain much lower amounts of 5'-demethoxy-5-MPT. In the case of the actual isolation, the methanol concentration was adjusted to 70%. The use of this more polar methanol concentration proved to give a more optimal separation as compared with 80%. When the use of this column was repeated three times, ca. 25% of the initial amount of 5-MPT was lost, however, the separation problem caused by the presence of 5'-demethoxy-5-MPT was solved.

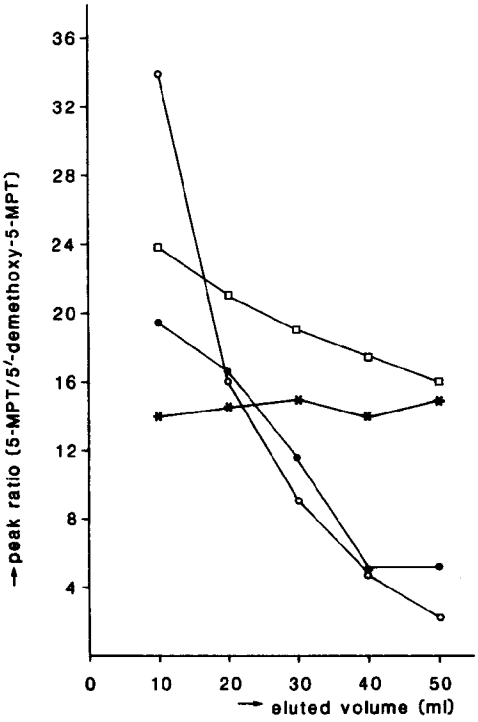

Fig. 3. Relationship between the peak ratio of 5-MPT/5'-demethoxy-5-MPT as detected with HPLC-UV at 290 nm and the elution volume, using different XAD-resins and a mobile phase consisting of 80% methanol; XAD-2 (✱), XAD-4 (□), XAD-7 (●) and XAD-8 (○).

The silica gel column with chloroform/acetone as the mobile phase was introduced to eliminate the yellow colour, which was present as an impurity in the crude 5-MPT. The choice of this mobile phase was based on the successful isolation of several lignans from *Juniperus sabina* (San Feliciano et al. 1990), including 5-MPT. This chromatography system proved to be very suitable to remove the yellow colour from the 5-MPT.

The 5-MPT was collected in the first fractions, while the coloured impurities were retained on the column. For further purification of the 5-MPT, another mobile phase was used, in combination with a silica gel column. The eluent was derived from the elution solvent as applied in the HPLC-system, namely: n-heptane:dichloromethane:methanol = 90:10:2.

This mobile phase was necessary to remove less polar, unknown, HPLC-UV detectable compounds with retention times between 1 and 8 min.
Rotary evaporation of the n-heptane/dichloromethane/methanol mixture containing 5-MPT, resulted in a white amorphous powder, as soon as the dichloromethane and methanol were disappeared. When 5-MPT was recovered after evaporation from dichloromethane, chloroform or methanol without the presence of n-heptane, the result was a transparant residue.
Various amounts of 5-MPT, in the range of 100-500 mg, present in extracts were subjected to the isolation procedure. No additional problems or diminished yields were observed when the initial amount of 5-MPT was increased from 100 to 500 mg, indicating that further upscaling should be feasible. The method developed, allowed the reuse of most solvents as well as the chromatographic materials. These advantages are of important economical value for the isolation of 5-MPT and limit environmental pollution.

CONFIRMATION OF THE IDENTITY

The isolated 5-MPT was subjected to a series of analytical methods in order to confirm its identity and check its purity. It was necessary to know whether the desired lignan, and in particular which enantiomer, was isolated in order to allow proper cytotoxicity experiments with the isolated 5-MPT and to use it as a starting compound for the chemical synthesis of derivatives.
The 5-MPT was for more than 99% pure on a HPLC basis. UV and IR-spectra revealed maxima, which corresponded fairly well with results reported earlier by San Feliciano et al. (1990) for 5-MPT isolated from *Juniperus sabina*. The mass spectrum contained fragments that were consistent with the literature MS-data on fragmentation patterns as reported previously for 5-MPT (Berlin et al. 1986; Broomhead and Dewick 1990; San Feliciano et al. 1990; Wichers et al. 1990). The determination of the melting point of 5-MPT gave problems, as no unambiguous melting point could be detected. A melting traject, between 95-100 °C, was found, while effervescence and foaming was observed at these temperatures. In addition, this melting traject was ca. 100 °C under the melting point as published by San Feliciano et al. (1990). This phenomenon may be explained by the fact that the crystallization of podophyllotoxin, itself, has variables. Recently, it has been reported that podophyllotoxin can be obtained in different crystal forms (Andersen et al. 1990). More importantly, several melting points or trajects have been found for podophyllotoxin. A melting point of 182-183 °C for the orthorhombic form, 162-164 °C for a trigonal hydrate and a melting traject between 114-118 °C with foaming for an orthorhombic solvate of water and benzene. It is quite reasonable to assume that 5-MPT is

also able to act as a host for certain solvent molecules, because the same hydrophylic and hydrophobic parts are present in both molecules.

Elemental analysis revealed a slight difference between the calculated and found % H value, 0.37% (absolute), and a smaller difference for the % C value, namely 0.23%. These differences indicate traces of a compound that is relatively rich of hydrogen. Probably, traces of n-heptane were present, since 5-MPT was finally obtained by evaporation of an n-heptane/dichloromethane/methanol mixture. In this way, a very stable guest-host complex must have been obtained, since it remained intact even after drying under low pressure or heating. The presence of traces n-heptane in a sample of pure 5-MPT could be confirmed by means of mass spectrometry.

Theoretically, of the sixteen stereoisomers that are possible, only one, analogous to podophyllotoxin, is of special interest, namely (-)-5-MPT. ^{1}H- and ^{13}C-NMR spectral data were compared with previously published data and clearly showed that the isolated lignan was the diastereomer 5-MPT (Berlin et al. 1986; Broomhead and Dewick 1990; San Feliciano et al. 1990; Wichers et al. 1990). To distinguish between (+)- and (-)-enantiomers, the optical rotation was measured, and it appeared that the desired lignan, with the correct configuration, had indeed been isolated. From comparison of the specific optical rotations (at λ = 589.3 nm, in chloroform) of podophyllotoxin and 5-MPT, being -132.0° and -129.5° respectively, it can be concluded that the additional methoxyl moiety at C-5 hardly influences the chirality of the molecule.

CYTOTOXICITY

In the present study, the cytotoxicity of the isolated 5-MPT against Ehrlich ascites tumour (EAT) and cervix uteri (HeLa) cells was determined and compared with those of 5-MPT-4-β-D-glucoside, podophyllotoxin, etoposide and teniposide.

Table 1. Cytotoxicity of the lignans as tested on 2 tumour cell lines. Data are expressed as ED_{50} values in μg ml^{-1}.

Compound	EAT[1]	HeLa[2]
5-MPT	32.0	22.0
podophyllotoxin	42.8	20.5
5-MPT-4-β-D-glucoside	30.0	21.8
etoposide	1.1	7.9
teniposide	0.06	0.3

1 murine Ehrlich ascites tumour cell line
2 human HeLa cervix uteri tumour cell line

From Table 1 it can be seen that 5-MPT, its glucoside and podophyllotoxin had about the same ED_{50} values in the experimental setting used. Berlin et al. (1988), on the contrary, found that the aglucone exhibited a 250-500 times higher cytotoxic activity than the 5-MPT-4-β-D-glucoside, as determined against murine fibroblast cells L929. The difference may be ascribed to the use of other test systems and to possible impurities of the tested compounds, since cell extracts were used in that study.

The mechanism of action of etoposide and teniposide, which is different from that of podophyllotoxin, resulted in a much stronger cytotoxic action against both tumour cell lines. The EAT cells were less sensitive than HeLa cells for 5-MPT, 5-MPT-4-β-D-glucoside and podophyllotoxin, while the opposite counts for etoposide and teniposide. Podophyllotoxin is a classical spindle poison, arresting cell division in the metaphase, a process which is connected with the inhibition of microtubule assembly (Stähelin and Von Wartburg 1991). Etoposide and teniposide do not show any effect on microtubule assembly, although their aglycones (4'-demethylated) behave like podophyllotoxin, but prevent cells from entering mitosis (Clark and Slevin 1987; Holthuis 1988; Van Maanen et al. 1988; Stähelin and Von Wartburg 1991). The cells are arrested in the late S or G_2 phase of the cell cycle. The exact mechanism of action of etoposide and teniposide is yet unknown, although it is obvious that the induction of DNA strand breaks by etoposide can be explained by the ability to inhibit nuclear topoisomerase II (Van Maanen et al. 1988). Essential for the change from spindle poison to G_2 poison are demethylation at position 4', epimerization at position 4 and presence of a glucose moiety at position 4, which has been condensed with an aldehyde (Van Maanen et al. 1988; Stähelin and Von Wartburg 1991). Consequently, 5-MPT and 5-MPT-4-β-D-glucoside are most likely spindle poisons and not G_2 poisons.

FINAL CONCLUSIONS

In this study, it has been shown that isolations of (-)-5-MPT from root-like cultures of *L. flavum* are possible and that 5-MPT is a potential cytotoxic compound. As already mentioned, podophyllotoxin and 5-MPT are chemically and physically closely related, and, since both compounds exhibit about the same cytotoxic behaviour in our test systems, 5-MPT might become a starting compound for the synthesis of new anti-tumour drugs. In analogy with podophyllotoxin from which etoposide and teniposide are prepared, the chemical synthesis of 4'-demethyl-5-methoxy-epipodophyllotoxin-ethylidene-β-D-glucoside and 4'-demethyl-5-methoxy-epipodophyllotoxin-thenylidene-β-D-glucoside, being the 5-methoxy variants of etoposide and teniposide, respectively, is under current investigation.

ACKNOWLEDGEMENTS

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CHAPTER 9

EPILOGUE

From the results obtained from our studies, it may be concluded that a biotechnological production of podophyllotoxin and related compounds by plant cell cultures is essentially possible. Since the main aim of our work was to investigate whether a biotechnological approach of production may overcome the problems in the commercial supply of these lignans, the production capacities of the plant cell cultures involved are discussed in this chapter.

The first part of the studies resulted in the establishment of plant cell cultures derived from three different plant species, that were all able to produce podophyllotoxin or related lignans. Cultures of *Podophyllum hexandrum* accumulated podophyllotoxin at a production rate of 1.3 mg l^{-1} day^{-1} (Chapter 2), those of *Linum flavum* produced 5-methoxypodophyllotoxin at a rate of 4.8-6.0 mg l^{-1} day^{-1} (Chapter 3), while cultures of *Callitris drummondii* synthesized podophyllotoxin-β-D-glucoside at a rate of 0.4 mg l^{-1} day^{-1} (Chapter 4). Since the latter cultures exhibited a low production rate, they were not involved in further studies.

Attempts were made to improve the production of lignans by means of elicitation, precursor feeding to freely suspended and entrapped cells, the optimization of production media and the induction/selection of differentiated cells, *i.e.* root-like tissue. For cultures of *P. hexandrum* the production rates could only be enhanced by precursor feeding to freely suspended cells. The main problem here was that the accumulation levels of podophyllotoxin under standard conditions were variable: they decreased dramatically while maintening the cultures for a longer period. The effect of precursor feeding was most evident in low-producing cultures. Feeding coniferin to cultures with the relatively high production rate of 0.5 mg podophyllotoxin l^{-1} day^{-1} resulted in an enhanced rate of 1.2 mg l^{-1} day^{-1}, while cultures with the very low rate of 0.02 mg l^{-1} day^{-1} started to produce at a rate of 0.8 mg l^{-1} day^{-1}, which may be considered as a giant increase.

The production of 5-methoxypodophyllotoxin by cultures of *L. flavum* could be improved by the induction/selection of differentiated cells as such as well as in combination with the use of different production media with or without precursors (Chapter 7, Table 2). The highest production rate obtained was 8.9 mg l^{-1} day^{-1} for root-like cultures.

The criteria that have to be met for the industrial production of secondary metabolites by plant cell cultures are not absolutely clear. During the last decade several reports dealing

with this subject have been published. The general problems associated with the commercial use of plant cell cultures is discussed in the following.

Generally, authors use model systems for their cost analyses and since many assumptions have to be made, such calculations can only give an indication concerning the industrial applicability of plant cell cultures. The balance between the price of the desired secondary metabolite, its market volume and the manufacturing costs determines the ultimate production possibilities (Balandrin et al. 1985; Constabel 1990). By scientists, a proper judgement of this balance can hardly be made, as the market volume and real product price are unstable parameters or, may even be unknown. The manufacturing costs for the industrial application of plant cell cultures are high and, in particular, the large-scale cultivation is accompanied with difficulties (Ellis 1986). There are significant engineering problems, mainly due to the slow growth, that requires precautions against contaminations and, in many cases, the fragility of plant cells. The fermentation hardware, routinely used for micro-organisms, is not optimal or not suitable at all, in large-scale operations involving plant cell cultures. This results in an additional investment for the development of novel or modified bioreactors (Shuler 1981; Ellis 1986).

For a number of plant cell cultures that have been described to accumulate secondary metabolites at high levels, we calculated their production rates (Table 1).

Table 1. Production rates of some high-producing cell suspensions.

Product	Culture	Production rate ($mg\ l^{-1}\ day^{-1}$)	Reference
rosmarinic acid	*Anchusa officinalis*	400	De-Eknamul and Ellis 1985
	Coleus blumei	223	Zenk et al. 1977
L-DOPA	*Mucuna pruriens*	177	Pras 1987
coniferin	*Linum flavum*	127	Van Uden et al. 1991
shikonin	*Lithospermum erythrorhizon*	100	Fujita and Hara 1985
verbascoside	*Syringa vulgaris*	90	Ellis 1983
berberine	*Thalictrum minus*	31	Nakagawa et al. 1984

With the exception of shikonin, none of these secondary metabolites are produced on an industrial scale. Even at relatively high production rates, the manufacturing costs are too high for the realization of a profitable process. It seems that the highest production rates are found for compounds with a chemical structure that is not too complex, such as L-DOPA, rosmarinic acid, coniferin and shikonin. Probably, more simple pathways are more readily expressed in *in vitro* circumstances.

With respect to the improvement of the *in vitro* production of lignans, the results are rather disappointing. The highest rates found for the production of podophyllotoxin by cell cultures of *P. hexandrum* were ca. 1 mg l^{-1} day^{-1}. These rates are too low for production on an industrial scale. However, it has to be taken into account that the research effort spent on the podophyllotoxin-product group is low compared with the efforts that have been spent on the other compounds from Table 1.

The annual demand for podophyllotoxin in The Netherlands is approx. 10 kg. No manufacturer's prices were available, but a price of ca. 400,000 Dutch guilder per kg podophyllotoxin is calculated for 500 mg quantities, based on a low catalogue price (Sigma Chemical Company). In the period 1987-1988, the annual turnover in The Netherlands for etoposide and teniposide came to 2.5 million Dutch guilder and has strongly increased in comparison with the former period. The turnover for the two cytostatics in the same period in both Europe and the USA, was estimated to be 50 million US dollar (Dr. Ir. C.B. Lugt, ACF Beheer B.V., Maarssen, The Netherlands, personal communication).

From this information it can be concluded that it remains worthwile to search for alternative production routes for podophyllotoxin with the aim to substitute the present, limited source for this lignan, the plants of *Podophyllum*. Only after further improvement of the production, plant cell cultures may be used to gain podophyllotoxin.

For the production of 5-methoxypodophyllotoxin by cell cultures of *L. flavum* a maximum rate of ca. 9 mg l^{-1} day^{-1} was calculated. 5-Methoxypodophyllotoxin is a new lignan. It may be used for the development of new anti-tumour compounds, that possibly act more specifically and are less toxic than the derivatives of podophyllotoxin, etoposide and teniposide. In addition, the possibility to remove the 5-methoxy moiety of 5-methoxypodophyllotoxin in order to obtain podophyllotoxin will be investigated. Recently, also the presence of other lignans, such as 5'-demethoxy-5-methoxypodophyllotoxin, has been demonstrated in cell cultures of *L. flavum* (Wichers et al. 1991). Future research will be focussed on the improvement of the production of lignans that are related to podophyllotoxin, particularly 5-methoxypodophyllotoxin, by selection of high-producing cells. An alternative to production by means of tissue culture could be the propagation of suitable plants through agriculture. Therefore, regeneration experiments using these selected cells will be performed.

After isolation, the cytotoxicity of the lignans will be tested *in vitro*, either as such or after chemical modification. If the results with respect to cytotoxicity of the lignans seem promising, larger-scale cultivation of *L. flavum* plants in the field, next to scaling-up of high producing plant cell cultures, should be regarded as well.

During the last two decades, it has been proven that plant cell cultures are able to produce secondary metabolites, even of significant structural complexicity (Berlin 1988). The power of plant cells is that they are able to form compounds, that can not be produced by micro-organisms or that are difficult to prepare by chemical synthesis, e.g. podophyllotoxin and related compounds. Moreover, many metabolites are synthesized stereospecifically. For instance, our cultures of *L. flavum* produced exclusively the enantiomer (-)-5-methoxypodophyllotoxin. For these reasons, further research on the *in vitro* production of secondary metabolites using plant cell cultures remains a challenge.

Despite intensive research, commercially seen, no appropriate methodology for the large-scale production of drugs using plant cell cultures has been established yet (Constable 1988; Tyler 1988). The best results have been obtained by use of more differentiated cultures, such as roots or shoots (Berlin 1988; Rother 1989; Strauss 1989). Nevertheless, it may be concluded that our knowledge regarding the manipulation of the expression of a desired secondary pathway is insufficient yet. These problems however, may be solved after more insight in the enzymology and molecular biology of secondary pathways has been obtained.

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SUMMARY

Podophyllotoxin is a lignan that occurs in several plant species. Its cytotoxic activity is well-known, but it can not be applied clinically because of severe toxic side effects. Attempts have been undertaken to develop less toxic and more specifically acting anti-tumour drugs derived from podophyllotoxin. Presently, etoposide and teniposide are clinically applied chemotherapeutics that are especially effective against testicular and small cell lung cancer. Podophyllotoxin is the starting compound for the chemical synthesis of these two cytostatics. Although several methods for a total chemical synthesis of podophyllotoxin have been reported, the foreseeable future will probably not yield a commercially attractive synthesis. Therefore, the availability of podophyllotoxin depends on its presence in the plant. However, the availability of *Podophyllum* plants, the best natural source of podophyllotoxin, has its limitations: the occurrence of the plants is scarce, they have a long juvenile phase and poor reproduction capacities. *Podophyllum hexandrum* has even become a threatened species due to the intense collection and the lack of organized cultivation. The biotechnological production of podophyllotoxin and related compounds is considered to be an alternative. Investigations in that direction are described in this Ph.D. thesis.

Chapter 1 is a general introduction. It deals with the history, the occurrence in plants, the (bio)synthesis and the mechanism of action of podophyllotoxin. In addition, the chemical synthesis, the mechanism of action and the clinical application of etoposide and teniposide are described. Furthermore, the current state on the availability of podophyllotoxin and the possibilities to produce lignans using plant cell cultures are discussed.

In Chapter 2, the initiation of cell cultures derived from the roots of *Podophyllum hexandrum* (Indian Podophyllum) is described. Undifferentiated callus cultures were found to accumulate podophyllotoxin. Generally, contents between 0 and 0.1% on a dry weight basis (DW) were measured. The highest content found was 0.3% (DW) in dark-grown callus. Cell suspension cultures also accumulated podophyllotoxin. The highest content determined in these cultures was 0.1% (DW) and was found during the stationary phase of the growth cycle. These dark-grown suspension cultures accumulated 3 to 4 times more podophyllotoxin, in comparison with light-grown cultures.

In Chapter 3, the initiation of cell cultures of *Linum flavum* (yellow flax) is described. Low levels, 0.015% (DW), of 5-methoxypodophyllotoxin (5-MPT) were accumulated in light-grown cell suspensions. Attempts were made to find a relationship between the 5-MPT production and the activity of phenylalanine ammonialyase (PAL), a key-enzyme in its biosynthesis. Treatment of the cultures with the elicitor Nigeran caused the 5-MPT production to cease, even though PAL activity was rapidly enhanced. Feeding experiments

with the phenylpropanoid L-phenylalanine, an early intermediate in the lignan biosynthesis, resulted in a 3-5 fold increase in 5-MPT levels, but caused the levels of PAL activity to fall. A far more efficient method to increase the 5-MPT production was the transfer of standard-grown cultures to phytohormone-free medium. This resulted in more differentiated cultures with a 40-50 fold higher 5-MPT content, 0.58-0.73% (DW), and again PAL activity levels became reduced as compared with the routinely-grown cells. L-Phenylalanine-feeding to the more organized cells had no effect on the 5-MPT contents, while elicitor treatment, on its own and in combination with the precursor, caused the 5-MPT production to cease again. Under all these experimental conditions using NAA-lacking cell cultures, the PAL-activities were higher than of the untreated culture. No general correlation could be found between the PAL-activities and the 5-MPT contents.

In Chapter 4, the initiation of suspension cultures derived from the needles of *Callitris drummondii* (Drummond's cypress pine) is described. Dark-grown cell suspensions contained approx. 0.02% (DW) podophyllotoxin, of which 85-90% was present in its β-D-glucosidic form. Illumination stimulated the endogenous production of podophyllotoxin (-β-D-glucoside); contents up to 0.11% (DW) were measured.

The first part of this Ph.D. study resulted in the establishment of cell cultures derived from three different plant species, that all accumulated podophyllotoxin or related lignans. Since cultures of *C. drummondii* exhibited a slow growth, resulting in low production rates, it was decided in further investigations to focus on the cell cultures of *P. hexandrum* and *L. flavum*.

In Chapter 5, further research on the improvement of the podophyllotoxin production by phenylpropanoid precursor feeding to cell cultures of *P. hexandrum* is presented. Eight phenylpropanoids; L-phenylalanine, L-tyrosine, *trans*-cinnamic acid, *trans*-caffeic acid, *trans*-ferulic acid, *trans*-coumaric acid, *trans*-3,4-methylenedioxycinnamic acid, and coniferin (coniferyl alcohol-β-D-glucoside) were fed to these cultures. Only upon addition of coniferin, a stimulation of the podophyllotoxin accumulation was observed. A 4.5-fold increase was found, namely from 0.018% to 0.085% (DW). Entrapment of the *P. hexandrum* cells in calcium alginate as such, or in combination with the feeding of phenylpropanoids, did not lead to an improved podophyllotoxin production. Permeabilization with isopropanol, in order to make the cells more accessible for coniferin, did not result in an additional increase in podophyllotoxin accumulation. Concentrations of isopropanol exceeding 0.5% (v/v) were found to be toxic for the cells. Under these conditions podophyllotoxin was released into the medium.

Coniferyl alcohol, the aglucone of coniferin, is a key-intermediate in the biosynthetic pathway of podophyllotoxin, but is poorly soluble in water (max. 0.15 mM). To enhance the water solubility of coniferyl alcohol, the use of β-cyclodextrin, as a clathrating agent

was introduced. By complexation with β-cyclodextrin, a solution of 3 mM coniferyl alcohol could be fed, resulting in a ca. 6-fold enhanced podophyllotoxin accumulation. The same concentration of non-complexed, suspended, coniferyl alcohol had 3-fold less effect on the podophyllotoxin accumulation. β-Cyclodextrin itself was proven to be non-toxic for the cells. The effect of coniferin, however, on the podophyllotoxin accumulation, was much stronger (ca. 5-fold) than that of coniferyl alcohol complexed with β-cyclodextrin. Because the podophyllotoxin accumulation in *P. hexandrum* cell suspensions appeared to be variable and lower contents (production rates) were measured after a longer period of maintenance, it was decided to concentrate further studies on the production of 5-MPT by cell cultures of *L. flavum*.

In Chapter 6, a cell line of *L. flavum* is presented that contains low levels of 5-MPT, but on the contrary, high levels of coniferin. Generally, low 5-MPT levels corresponded with high coniferin contents, and vice versa. Cell suspension, callus and leaves were compared for metabolite accumulation and associated enzyme activities. Cell suspension cultures accumulated up to 12.4% coniferin (DW), callus cultures contained 5.6%, while no coniferin could be detected in leaves of *in vitro*-grown plantlets. The 5-MPT levels in these materials were 0.014%, 0.035% and 0.100% (DW), respectively. A reciprocal relationship between β-glucosidase activity and coniferin accumulation was found. No relationship between peroxidase activity and coniferin or 5-MPT accumulation could be demonstrated. Finally, a rapid and efficient isolation procedure for coniferin, which is not commercially available, is described in this chapter.

In Chapter 7, a selected root-like cell line of *L. flavum* was used to optimize the composition of the growth medium with respect to the production of 5-MPT. The omittance of the phytohormone naphthalene acetic acid (NAA) from the medium resulted in further root formation, which was accompanied with a 2,6-fold increase of 5-MPT levels. The feeding of the phenylpropanoids L-tyrosine or L-phenylalanine resulted in further enhanced 5-MPT contents. After omittance of the vitamins together with the phytohormone, 5-MPT levels became 6-fold enhanced. The maximal content ever found applying this production medium was 1.01% (DW). The use of higher sucrose concentrations led to a proportional biomass increase. Cells grown on 6% sucrose yielded 121.4 mg l^{-1} 5-MPT.

In order to obtain pure 5-MPT from this root-like culture of *L. flavum*, an isolation procedure was developed, that is described in Chapter 8. The occurrence of the structurally strongly resembling lignan 5'-demethoxy-5-MPT, was the main cause for problems in isolating pure 5-MPT. Important steps in the isolation procedure were: dichloromethane extraction, XAD-4 adsorption, and XAD-8 column chromatography followed by silica gel chromatography, using two different mobile phases. The ultimately

isolated 5-MPT was >99% pure and possessed the desired stereochemical configuration, namely (-)-5-MPT. The *in vitro* cytotoxicity of the isolated 5-MPT against Ehrlich ascites and HeLa cervix uteri tumour cell lines was determined and compared with 5-MPT-4-β-D-glucoside, podophyllotoxin, etoposide and teniposide. 5-MPT exhibited approximately the same cytotoxicity as podophyllotoxin. Therefore, 5-MPT might become a starting compound for the synthesis of new anti-tumour drugs. In analogy with podophyllotoxin, from which etoposide and teniposide are prepared, the chemical synthesis of 4'-demethyl-5-methoxyepipodophyllotoxin-ethylidene-β-D-glucoside and 4'-demethyl-5-methoxyepi-podophyllotoxin-thenylidene-β-D-glucoside, being the 5-methoxy variants of etoposide and teniposide, respectively, are under current investigation.

In Chapter 9, an epilogue, the production rates of the three plant cell cultures under the different experimental conditions are compared with each other and with high-producing cell cultures, that are known from literature. Finally, the perspectives for the biotechnological production of lignans using plant cell cultures is discussed.

SAMENVATTING

Podofyllotoxine is een lignaan dat in verschillende plantesoorten voorkomt. De verbinding is cytotoxisch, maar kan niet in de kliniek worden toegepast vanwege de ernstige bijwerkingen. Er zijn pogingen ondernomen om minder giftige en meer specifiek werkende antitumorverbindingen te ontwikkelen met podofyllotoxine als uitgangsverbinding. Etoposide en teniposide worden tegenwoordig in de kliniek toegepast als chemotherapeutica, die in het bijzonder effectief zijn gebleken bij de behandeling van testiskanker en kleincellig longkanker. Podofyllotoxine is de startverbinding voor de chemische bereiding van beide cytostatica. Alhoewel verschillende methoden zijn beschreven voor een chemische totaal-synthese van podofyllotoxine, zal de nabije toekomst hoogst waarschijnlijk geen commercieel aantrekkelijke synthese opleveren. Daarom hangt de beschikbaarheid van podofyllotoxine af van de aanwezigheid in de plant. Echter, de beschikbaarheid van *Podophyllum* planten, de beste natuurlijk bron, is beperkt: de planten komen slechts schaars voor, hebben een lange juveniele fase en zijn slecht te vermeerderen. Eén soort, *Podophyllum hexandrum*, wordt zelfs bedreigd in zijn voortbestaan, vanwege de intensieve verzameling uit het wild en het achterwege blijven van een georganiseerde cultivatie. De biotechnologische produktie van podofyllotoxine en daaraan gerelateerde verbindingen wordt beschouwd als een alternatief. Onderzoek in deze richting wordt in dit proefschrift beschreven.

Hoofdstuk 1 is een algemene introduktie. De historie, het voorkomen in planten, de (bio)synthese en het werkingsmechanisme van podofyllotoxine worden behandeld. Bovendien wordt ingegaan op de chemische synthese, het werkingsmechanisme en de klinische toepassing van etoposide en teniposide. Verder worden de huidige stand van zaken ten aanzien van de beschikbaarheid van podofyllotoxine en de mogelijkheid om lignanen te produceren met behulp van plantecelcultures besproken.

In Hoofdstuk 2 wordt de initiatie van celcultures uit de wortels van *Podophyllum hexandrum* (Indiase Podophyllum) beschreven. Ongedifferentieerde calluscultures bleken in staat podofyllotoxine te accumuleren. Gehaltes tussen 0 en 0,1% op basis van het drooggewicht (DW) werden gemeten. Het hoogste gehalte werd gevonden in callus, die in het donker werd gekweekt, namelijk 0,3% (DW). Ook de celsuspensies bleken in staat te zijn om podofyllotoxine te produceren. Maximaal 0,1% (DW) werd gevonden in cellen, die zich in de stationaire fase van de groeicyclus bevonden. Deze in het donker groeiende cultures bevatten 3-4 maal meer podofyllotoxine dan de cultures die in het licht groeiden.

In Hoofdstuk 3 wordt de initiatie beschreven van celcultures uit de bladeren van *Linum flavum* (gele vlas). In de celsuspensies, die in het licht groeiden, werden lage gehaltes, 0,015% (DW), gevonden van het 5-methoxypodofyllotoxine (5-MPT). Fenylalanine

ammonialyase (PAL) is een sleutelenzym in de biosyntheseroute van lignanen. Daarom werd getracht een relatie te vinden tussen de aktiviteit van PAL en de produktie van het 5-MPT. Behandeling van de cultures met de 'elicitor' Nigeran, resulteerde in het stoppen van de produktie van het 5-MPT, ofschoon de PAL aktiviteit zeer snel werd verhoogd. Experimenten waarbij L-fenylalanine, een intermediair in de lignaanbiosynthese, werd toegevoegd aan de celsuspensies, gaven 3-5 maal verhoogde 5-MPT-gehaltes, maar een verlaagde PAL-aktiviteit. Een veel efficiëntere methode om de produktie van 5-MPT te verhogen was het overbrengen van de culture, die gekweekt werd onder standaardcondities, naar hormoonvrij medium. Dit resulteerde in meer gedifferentieerde cultures met 40-50 maal hogere 5-MPT-gehaltes, namelijk 0,58%-0,73% (DW), terwijl de PAL aktiviteit wederom werd verlaagd. Het toevoegen van L-fenylalanine aan deze cultures had geen effect op de produktie van 5-MPT, terwijl behandeling met de 'elicitor', al dan niet in combinatie met deze voorloper, opnieuw resulteerde in het stoppen van de produktie van het 5-MPT. In al deze experimenten, waarbij het medium geen hormoon bevatte, waren de PAL-aktiviteiten hoger dan van de onbehandelde culture. Er kon geen algemeen verband worden gevonden tussen de PAL-aktiviteit en het 5-MPT-gehalte.

In Hoofdstuk 4 wordt de initiatie besproken van celsuspensies afgeleid van naalden van de conifeer *Callitris drummondii* (Drummond cypres). Cultures die in het donker groeiden bevatten 0,02% (DW) podofyllotoxine, waarvan 85-90% aanwezig was als podofyllotoxine-β-D-glucoside. Licht stimuleerde de endogene produktie van podofyllotoxine(-β-D-glucoside); gehaltes tot 0,11% (DW) werden gemeten.

Het eerste gedeelte van dit promotieonderzoek resulteerde in het verkrijgen van celcultures van drie verschillende plantesoorten, die allen in staat bleken podofyllotoxine of daaraan gerelateerde lignanen te accumuleren. Daar celsuspensies van *C. drummondii* erg traag groeiden, resulterend in lage produktiesnelheden, werd besloten om verder onderzoek toe te spitsen op cultures van *P. hexandrum* en *L. flavum*.

In Hoofdstuk 5 wordt getracht de produktie van podofyllotoxine te verbeteren door het toevoegen van fenylpropanen aan celsuspensies van *P. hexandrum*. Acht fenylpropanen, te weten, L-fenylalanine, L-tyrosine, *trans*-kaneelzuur, *trans*-koffiezuur, *trans*-ferulazuur, *trans*-coumaarzuur, *trans*-3,4-methyleendioxykaneelzuur en coniferine (coniferylalcohol-β-D-glucoside) werden toegevoegd aan deze cultures. Alleen na toevoeging van coniferine werd de produktie van podofyllotoxine gestimuleerd. Het podofyllotoxinegehalte werd maximaal met een faktor 13 verhoogd tot 0,077% (DW). De immobilisatie van de *P. hexandrum* cellen in calciumalginaat, al dan niet in combinatie met de toevoeging van fenylpropanen, leidde niet tot een verbetering van de podofyllotoxineproduktie. Permeabilisatie met behulp van isopropanol, om de cellen meer toegankelijk te maken voor de aangeboden coniferine, resulteerde niet in een extra produktie van podofyllotoxine.

Bovendien bleken isopropanolconcentraties die hoger waren dan 0,5% (v/v), toxisch voor de cellen. Onder deze omstandigheden werd podofyllotoxine in het medium uitgescheiden. Coniferylalcohol, het aglucon van coniferine, is een belangrijke voorloper in de biosynthese van podofyllotoxine, maar is slecht oplosbaar in water (max. 0,15 mM). Door gebruik te maken van β-cyclodextrine, dat een wateroplosbaar complex vormt met het coniferylalcohol, kon de oplosbaarheid worden verhoogd tot 3 mM. Het toevoegen van dit complex aan een celsuspensie van *P. hexandrum* resulteerde in een 6 maal verhoogde podofyllotoxine-accumulatie. Dezelfde concentratie van gesuspendeerd (ongecomplexeerd) coniferylalcohol had 3 maal minder effect op de podofyllotoxine-accumulatie. β-Cyclodextrine zelf bleek niet toxisch te zijn voor de plantecellen. Het effect van coniferine op de podofyllotoxine-accumulatie was echter veel sterker (ca. 5 maal) dan van het coniferylalcohol/β-cyclodextrine complex.

Omdat de accumulatie van podofyllotoxine in *P. hexandrum* celsuspensies, gedurende verschillende experimenten, variabel bleek en steeds lagere gehaltes (produktiesnelheden) werden gemeten naarmate de cellen langer in kweek werden gehouden, werd besloten om in vervolgstudies uitsluitend gebruik te maken van celcultures van *L. flavum*.

In Hoofdstuk 6 wordt een cellijn van *L. flavum* gepresenteerd, die lage 5-MPT-concentraties bevat, maar daarentegen, hoge coniferine-gehaltes. In het algemeen correspondeerden lage 5-MPT-gehaltes met hoge coniferine-gehaltes en andersom. Celsuspensies, callus en blad werden vergeleken wat betreft metabolietaccumulatie en twee daarin betrokken enzymaktiviteiten. Celsuspensies bevatten coniferinegehaltes van 12,4% (DW), callus cultures 5,6%, terwijl géén coniferine werd aangetoond in de bladeren van *in vitro* gekweekte plantjes. De 5-MPT-gehaltes in hetzelfde materiaal waren respectievelijk 0,014%, 0,035% en 0,100% (DW). Het verband tussen de β-glucosidase-aktiviteit en de coniferine-accumulatie bleek omgekeerd evenredig te zijn. Er kon geen relatie tussen de peroxidase-aktiviteit en de coniferine- of 5-MPT-produktie worden aangetoond.

Tenslotte wordt in dit hoofdstuk een snelle en efficiënte isolatieprocedure voor coniferine, dat in de handel niet verkrijgbaar is, beschreven.

In Hoofdstuk 7 wordt een geselecteerde wortelculture van *L. flavum* gebruikt in experimenten om tot een geschikt medium te komen voor een maximale produktie van het 5-MPT. Het weglaten van het fytohormoon naftaleenazijnzuur uit het medium leidde tot een sterkere wortelontwikkeling, hetgeen gepaard ging met 2,6 maal verhoogde 5-MPT-gehaltes. De toevoeging van de fenylpropanen L-tyrosine of L-fenylalanine resulteerde in een verdere verhoging van de 5-MPT-gehaltes.

Het weglaten van het fytohormoon én de vitamines gaf een 6 maal hogere 5-MPT-accumulatie. Het hoogste gehalte, dat ooit werd gevonden door gebruik te maken van dit produktiemedium, was 1,01% (DW). Het gebruik van hogere sucroseconcentraties gaf

een evenredige toename van de biomassa. Cultures, die groeiden op een medium met 6% sucrose, produceerden 121,4 mg l^{-1} 5-MPT.

Met als doel zuiver 5-MPT te isoleren uit deze wortelculture werd een isolatieprocedure ontwikkeld, die wordt beschreven in Hoofdstuk 8. De aanwezigheid van het structureel sterk verwante 5'-demethoxy-5-MPT gaf de meeste problemen bij de isolatie van het 5-MPT. Belangrijke stappen in de isolatieprocedure waren: dichloormethaanextractie en XAD-4-adsorptie, alsmede kolomchromatografie met XAD-8 gevolgd door kolomchromatografie met behulp van silicagel, waarbij twee verschillende loopvloeistoffen werden gebruikt.

Het uiteindelijk geïsoleerde 5-MPT was voor meer dan 99% zuiver en bezat de gewenste stereochemische configuratie, namelijk (-)-5-MPT.

Met behulp van Ehrlich ascites- en HeLa cervix uteri tumorcellijnen werd de *in vitro* cytotoxiciteit van het geïsoleerde 5-MPT bepaald en vergeleken met 5-MPT-4-β-D-glucoside, podofyllotoxine, etoposide en teniposide. Het 5-MPT bleek een vergelijkbare cytotoxiciteit te bezitten als podofyllotoxine. Daarom kan 5-MPT mogelijk dienen als uitgangsverbinding voor de synthese van nieuwe antitumorverbindingen. Analoog aan de bereiding van etoposide en teniposide uit podofyllotoxine, worden de synthesemogelijkheden van 4'-demethyl-5-methoxyepipodofyllotoxine-ethylideen-β-D-glucoside en het 4'-demethyl-5-methoxyepipodofyllotoxine-thenylideen-β-D-glucoside, de 5-methoxy-varianten van respectievelijk etoposide en teniposide, momenteel onderzocht.

In Hoofdstuk 9, de epiloog, worden de produktiesnelheden van de drie plantecelcultures onder de verschillende experimentele condities met elkaar en met die vanuit de literatuur bekende hoogproducerende celcultures vergeleken. Tenslotte wordt ingegaan op de perspectieven voor de biotechnologische produktie van lignanen met behulp van plantecelcultures.

NAWOORD

Als je aan een promotiestudie begint, kun je moeilijk inschatten wat je uiteindelijk te wachten staat. In het eerste jaar rest er nog veel tijd en berichten dat het merendeel van de a.i.o.'s niet op schema ligt met het onderzoek, werken zeer geruststellend. In het tweede jaar begint er een paniekerig gevoel te ontstaan omdat de voortgang van het onderzoek inderdaad te langzaam gaat en de tijd te snel. Na deze vaststelling wordt reserve-energie aangesproken en in het derde jaar kan er 'geoogst' worden. Het vierde en laatste jaar is weliswaar druk, maar de zekerheid dat er genoeg studieresultaten zijn verzameld om een proefschrift samen te stellen, doet de psychische belasting verminderen. Uiteindelijk is het proefschrift voltooid en zijn alle inspanningen en teleurstellingen die nu eenmaal bij elk onderzoek horen, vergeten.

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De omslag bepaalt de eerste indruk van een boek. De artistieke steun van Wim Vaas heeft geresulteerd in een omslag waarmee de presentatie van dit proefschrift op verantwoorde wijze kan plaatsvinden.

Wim van Uden

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